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PATENT
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Printed name of person mailing correspondence

Susan M Cannon
Signature of person mailing correspondence

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Venkat Gopalan et al.	Art Unit:	1652
Serial No.:	09/516,061	Examiner:	Charles L. Patterson Jr.
Filed:	March 1, 2000	Customer No.:	21559
Title:	NOVEL BACTERIAL RNASE P PROTEINS AND THEIR USE IN IDENTIFYING ANTIBACTERIAL COMPOUNDS		

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. PAUL EDER UNDER 37 C.F.R. § 1.131

I, Paul Eder, declare that:

1. I am an inventor of the invention described and claimed in the above-identified patent application.

2. I received a doctorate degree in 1991 from the University of Iowa. I have been working in the field of RNase P biology for over 14 years. I was an employee of Message Pharmaceuticals from 1998 to 2004, first as a Project Leader and most recently as the Director of RNA Biology. I am now a Principal Scientist at Digene Corporation.

3. Claim 8, as amended in the reply to Office action submitted on June 24, 2004, recites a method of identifying an agent, which may be useful as an antibacterial agent. This method includes contacting an RNase P holoenzyme having a polypeptide with an RNase P consensus sequence with a compound and then measuring the enzymatic activity of the RNase P holoenzyme in the presence and absence of the compound. A compound is identified as an agent which may be useful as an antibacterial agent if it produces a detectable decrease in the enzymatic activity of RNase P as compared to the enzymatic activity in the absence of the compound.

4. I, along with the other inventors, conceived of the subject matter of claim 8 in the United States prior to January 7, 2000 and used reasonable diligence in reducing the invention to practice from prior to January 7, 2000 to the filing date of the application, March 1, 2000.

5. The conception and reduction to practice of the claimed invention is evidenced by Exhibit 1, annexed hereto. Exhibit 1 is a notebook entry, which begins prior to January 7, 2000 and continues up to the filing date of the application. These notebook pages show the design and development of an assay to measure RNase P activity in a 96-well plate format. This assay was designed for use as a screening assay for compounds that inhibit RNase P activity. This assay was used to test the inhibitory effects of neomycin B, a known antibiotic, as documented on pages 121 and 122 of the notebook entry.

Exhibit 1 documents that a method of identifying an antibiotic agent by screening for compounds that inhibit the enzymatic activity of an RNase P holoenzyme having a polypeptide with an RNase P consensus sequence, as described in the present application, was conceived of prior to January 7, 2000 and was diligently reduced to practice from prior to January 7, 2000 to the filing of the application on March 1, 2000.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 15 JULY 2004

PSEder

Paul S. Eder, Ph.D.
Principal Scientist
Digene Corporation

RNase P heterologous enzyme ~~se~~ (E. coli protein + S. aureus RNA) was incubated with 85 nt 5'-TAMRA labeled substrate for sufficient time for maximum cleavage. The products were separated on a PAGE gel, cut, and separated by crush and soak (The experiment was performed by Paul Eder).

The recovered RNA (20 µl) was diluted 10-fold in 10 mM MOPS (pH = 7.5), 150 mM KCl, 5 mM $MgCl_2$ and then diluted serially by two-fold

Sample →	85 mer	75 mer	10 mer
Conc.	(mP)	(mP)	(mP)
Initial	228.6	309	191.8
1/2	230.0	314	186.5
1/4	224.0	326	187.9
1/8	249.1	331	183.5
1/16	249.4	346	221.9
Avg →	236.0	325.2	194.3
S.D. →	11.8	14.6	15.7

There is a significant decrease in polarization (measured) on decreasing the size of the substrate, i.e., on cleavage. This decrease is sufficient to configure an assay. However, the product (75 mer) is fluorescent, thus in a homogeneous format the decrease in measured fluorescence would be significantly affected.

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Stock Concentrations:

E coli RNase P RNA, M1 = 8 pmol/µl

E. coli RNase P protein, CS = 20 pmol/µl

Substrate, pAT45-Cy3 = 160 pmol/µl

Renaturation:

- RNA needs to fold first and then needs to associate with protein
- CS is in urea and the urea has to be diluted by ~ 10-fold.

19 µl H_2O

3 µl 10x PA

3 µl M1

Then, heat @ 65°C for 5 minutes

heat @ 55°C for 5 minutes

heat @ 37°C for 5 minutes

Add 3 µl CS

Then, heat @ 37°C for 5 minutes

Place on ice.

Paul does the reaction at 37°C, by adding 2 µl of pAT45-Cy3. The CS protein is in excess during renaturation and during cleavage the substrate is in excess.

The RNaseP enzyme was renatured as prescribed above and an experiment set-up so that a variety of enzyme and substrate concentrations could be tested. The fluorometer was used in intensity mode with filters to detect TAMRA

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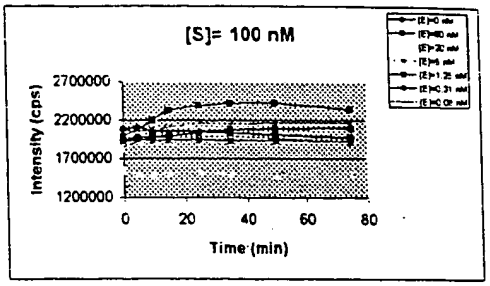
Work continued from Page 74

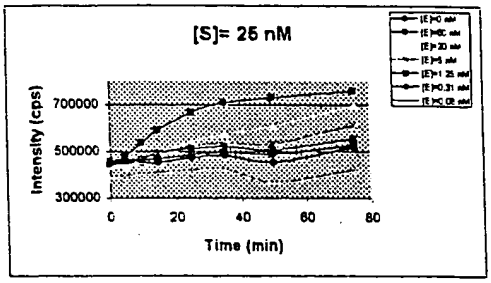
[S]=100 nM

Time (min)	[E]=0 nM	[E]=80 nM	[E]=20 nM	[E]=5 nM	[E]=1.25 nM	[E]=0.31 nM	[E]=0.08 nM
0	2080017.5	1876363	1540251	2048470	1906088.5	1623570	1832605.5
5	2082803	2062044	1489000.5	2036931.5	1924666	1853081.5	1901491.5
10	2036904.5	2173206	1483121	2043349	1916287	1870375.5	1948073.5
15	2044026	2285758	1517018.5	2084790	1924278	1879249	1946707.5
25	2032202	2369328	1526046	2136117.5	1927656.5	2022188	1957774.5
35	2051484.5	2389806	1500885.5	2131482	1916268.5	2016939.5	1972283
50	2065281	2398387	1481047.5	2145387	1919372	1995620.5	1937309.5
75	2078824	2317732	1497863	2146093	1906313	1957380	1906831.5



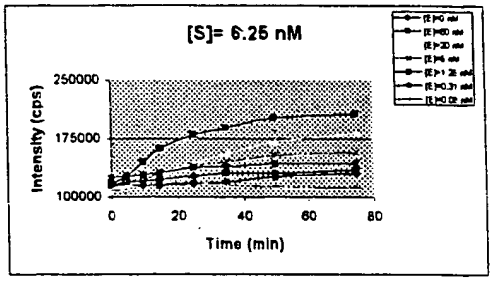
[S]=25 nM

Time (min)	[E]=0 nM	[E]=80 nM	[E]=20 nM	[E]=5 nM	[E]=1.25 nM	[E]=0.31 nM	[E]=0.08 nM
0	435859.5	435288	435526	454098	456288	439573.5	383430.5
5	445431	471098	431145	447669	461431	453383.5	391869
10	448288	530764.5	455002.5	460002.5	461812	458716.5	401335.5
15	446193	586574.5	493955	489717	469860	464717	404859
25	470335.5	661861	542622	517002.5	507622	481384	415716.5
35	476478.5	701575.5	577194	534479	515812.5	495526.5	420050
50	450669	723909	608194	526955	501907	482288	383430
75	513479	753871	695956.5	607956	549907.5	522193.5	418002.5



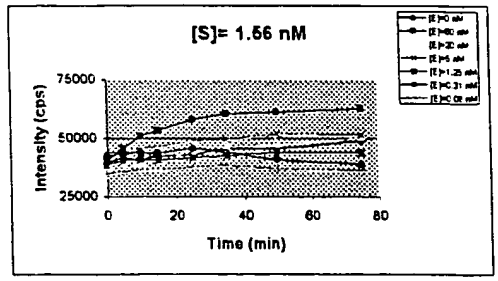
[S]=6.25 nM

Time (min)	[E]=0 nM	[E]=80 nM	[E]=20 nM	[E]=5 nM	[E]=1.25 nM	[E]=0.31 nM	[E]=0.08 nM
0	110952.5	114667	110952.5	118143	123334	113361	106524
5	114666.5	125905	113333.5	119571.5	124619.5	117429	108524
10	113000	143095.5	119762.5	121429	126810	118619.5	111524
15	113000	160381.5	129857.5	128381.5	129476.5	120810	114762.5
25	115524	178286.5	143476.5	138238.5	136334	125191	117000.5
35	117381	187667.5	156238.5	144000.5	137048	128809.5	114048
50	124476.5	200429.5	170953	152095.5	141381.5	129095.5	111143
75	128857.5	205905.5	182620	156286	140905	133288	109714.5



[S]=1.56 nM

Time (min)	[E]=0 nM	[E]=80 nM	[E]=20 nM	[E]=5 nM	[E]=1.25 nM	[E]=0.31 nM	[E]=0.08 nM
0	38762	39380.5	36238	43190.5	37762	41619	34285.5
5	40904.5	44809.5	37095	43571	39714	43142.5	35047.5
10	39904.5	50190	39333	45095.5	40428	43333	35856.5
15	41761.5	52571.5	39857	45809.5	40095	43047.5	35999.5
25	42476	57286	43285.5	48237.5	41190.5	44809.5	37047.5
35	45047	59762	46047.5	48904.5	42047.5	43285.5	37904.5
50	45333	60619	47047.5	51286	43428.5	40333	36380.5
75	48190	62095	48762	50952	43381	38428.5	35761.5



There is significant increase in intensity on addition of substrate. [S] = 6.25 nM and 25 nM, and [E] = 5 nM, 20 nM and 80 nM were chosen for further investigation.

Note: 1XPA is 50 mM Tris-HCl (pH = 7.5), 100 mM NH_4Cl, 15 mM $MgCl_2$, 10 mM DTT. In present experiment 100 mM NaCl was used in place of 100 mM NH_4Cl.

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The E.coli RNaseP was reactivated and reconstituted according to the protocol given by Paul Eder. Paul's 10xPA buffer was also used.

Fluorescence intensity and polarization using TAMRA settings were determined at each time point (due to time taken for the measurement) the time points are approximate.

[S]=25 nM

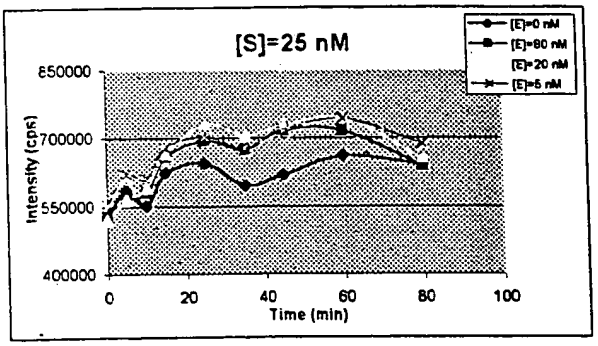
Time (min)	[E]=0 nM	[E]=80 nM	[E]=20 nM	[E]=5 nM
0	524490	525014	518919	552107.5
5	582915	561773.5	563916	615818
10	548250.5	578106	567390.5	606722.5
15	620055.5	655624.5	663338.5	676908.5
25	641530	691622	731762.5	700145.5
35	591914	671814	706526	675766
45	616151	715049.5	728810.5	713716
60	658005.5	713764	745142.5	739714.5
80	639720.5	636149.5	654815	665051

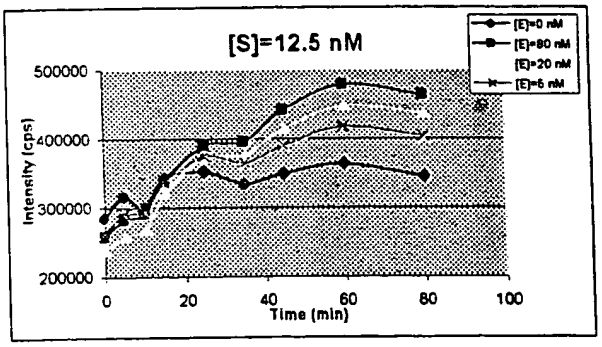
[S]=12.5 nM

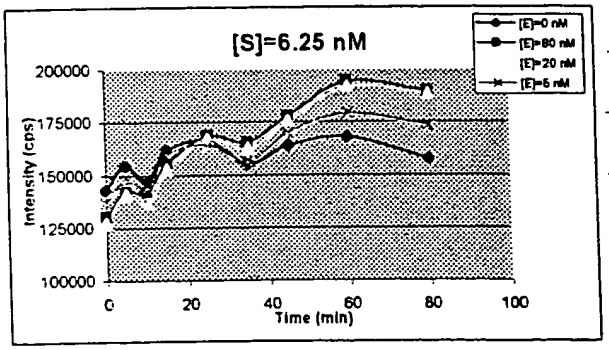
Time (min)	[E]=0 nM	[E]=80 nM	[E]=20 nM	[E]=5 nM
0	283886	256268.5	238841.5	261745
5	314598.5	280458	259364	289505
10	298171	290409.5	268744	292981
15	340930.5	336930.5	320979	333835.5
25	350453.5	388451	372785.5	372642.5
35	332359.5	394308	377213.5	363833.5
45	347977.5	439447.5	414211	387451.5
60	361405	476969	448066	415449.5
80	342787	461827	437257.5	401212

[S]=6.25 nM

Time (min)	[E]=0 nM	[E]=80 nM	[E]=20 nM	[E]=5 nM
0	142561.5	129753	127039	136657
5	153751.5	141562	140609.5	146609.5
10	146323.5	141133.5	136324	143324
15	161227.5	154942	152656.5	158989.5
25	165941	167941.5	166846	163703.5
35	154370.5	164989	162322.5	155799.5
45	163322.5	177226.5	175321.5	169084
60	167322.5	193796.5	191559	178417
80	156799	189321	188225.5	173036.5







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Work continued from Page 77

[S]=25 nM

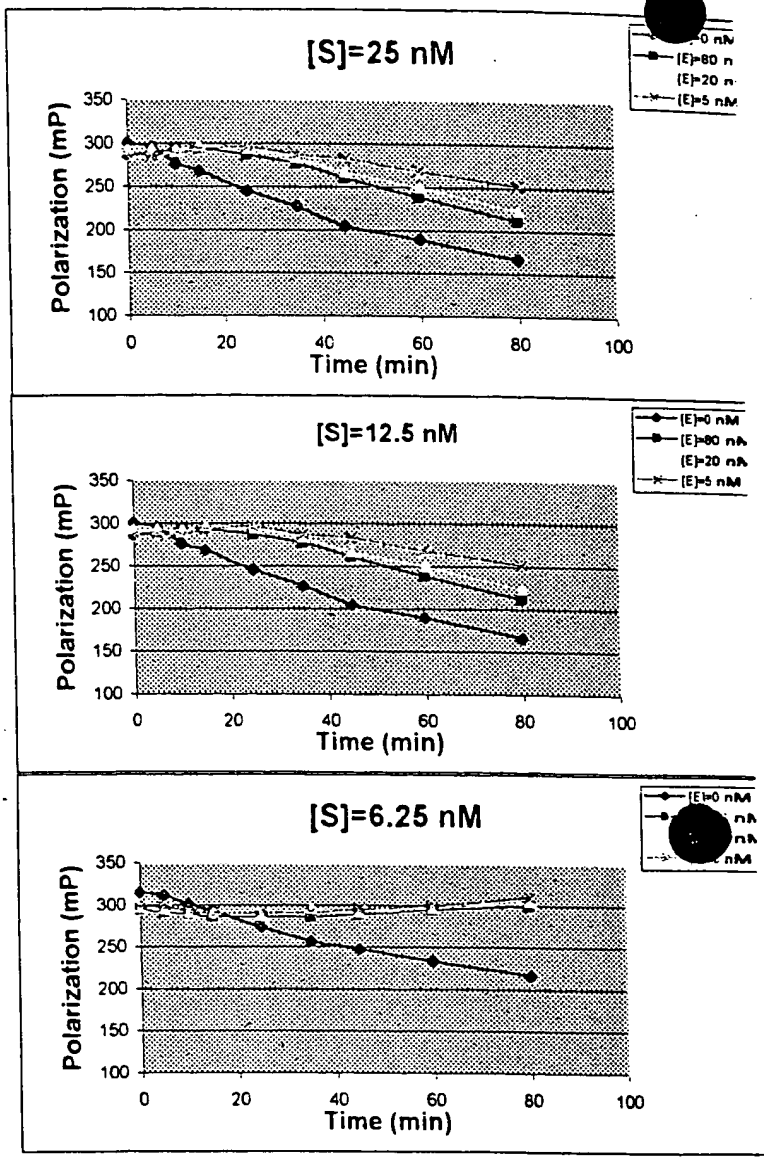
Time (min)	[E]=0 nM	[E]=80 nM	[E]=20 nM	[E]=5 nM
0	296.6	298.2	294.15	289.7
5	281.65	282.45	283.15	279.85
10	258.1	263.65	261.75	258.7
15	241.15	242.1	228.75	233.25
25	216.45	218.6	189.55	195.15
35	192.05	186.8	167.6	174.3
45	167.2	171.8	152.45	161.45
60	133.55	150.9	136.5	143.65
80	125.7	144.05	137.55	137.05

[S]=12.5 nM

Time (min)	[E]=0 nM	[E]=80 nM	[E]=20 nM	[E]=5 nM
0	299.15	284.85	292.45	284.75
5	294	287.6	291.75	284.7
10	275.1	292.3	291.2	285.15
15	267.15	292.7	296.05	291.1
25	243.8	285.25	293.6	294.65
35	225.55	275.55	283.3	285.7
45	203.55	259.5	266.55	281.5
60	187.85	236.9	249.85	266.7
80	165.4	210.25	223.25	250.8

[S]=6.25 nM

Time (min)	[E]=0 nM	[E]=80 nM	[E]=20 nM	[E]=5 nM
0	313.2	293.45	295.7	297.05
5	310.2	289.75	293.35	296.05
10	299.3	288.15	289.6	291.65
15	291.15	284.6	289.35	288.3
25	272.15	284.6	286.75	288.7
35	255.6	284.55	295.15	289.7
45	246.3	287.8	290.75	293.35
60	231.8	293.3	295.4	298.55
80	215.3	298.2	304.3	308.75



The inconclusive results above may be due to RNase inhibition of the substrate (probably from the buffer). I had detected this on a gel from the same substrate. Experiments will have to be repeated.

Work continued to Page

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TITLE Optimizing RNase P Assay Conditions

PROJECT NO.

BOOK NO.

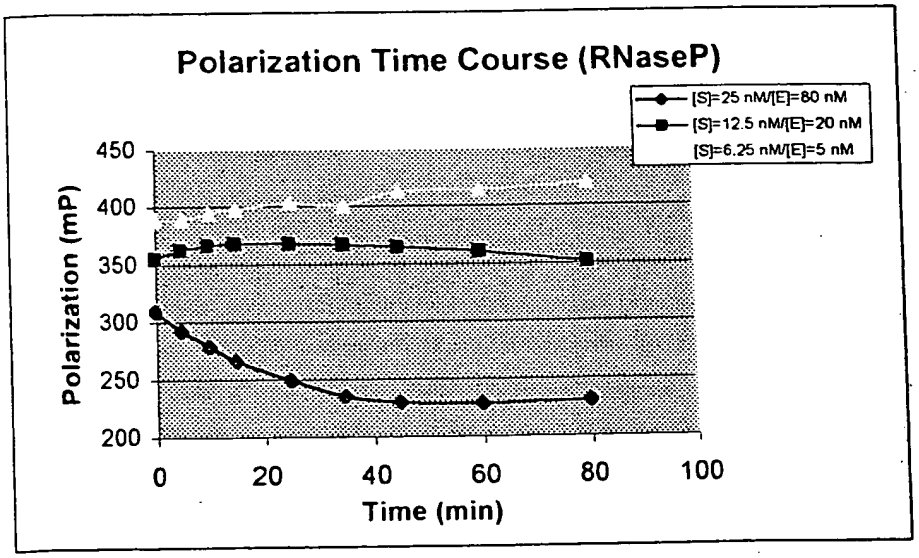
Work continued from Page ____

The RNase P enzyme was renatured according to Paul Eder's protocol, using new 10 X PA buffer, as there was suspected RNase contamination in the experiment done on 12/22-23/99.

Three different substrate : enzyme ratios were tried and the readings from eight different samples averaged. A time course was done and both polarization and intensity were measured with standard settings.

Time (min)	[S]=25 nM/[E]=80 nM	[S]=12.5 nM/[E]=20 nM	[S]=6.25 nM/[E]=5 nM
0	309.925	356.0125	389.2
5	292.7875	363.0571429	389.2
10	279.475	366.9625	394.1875
15	266.4375	368.95	398.0625
25	249.875	368.775	402.95
35	235.9	367.5125	400.875
45	229.9375	365.3625	412.45
60	228.9875	361.3625	414.15
80	231.175	352.3875	419.1

Data is average of eight different wells.



There was a good polarization change only for [S]=25nM/[E]=80nM

Work continued to Page 80

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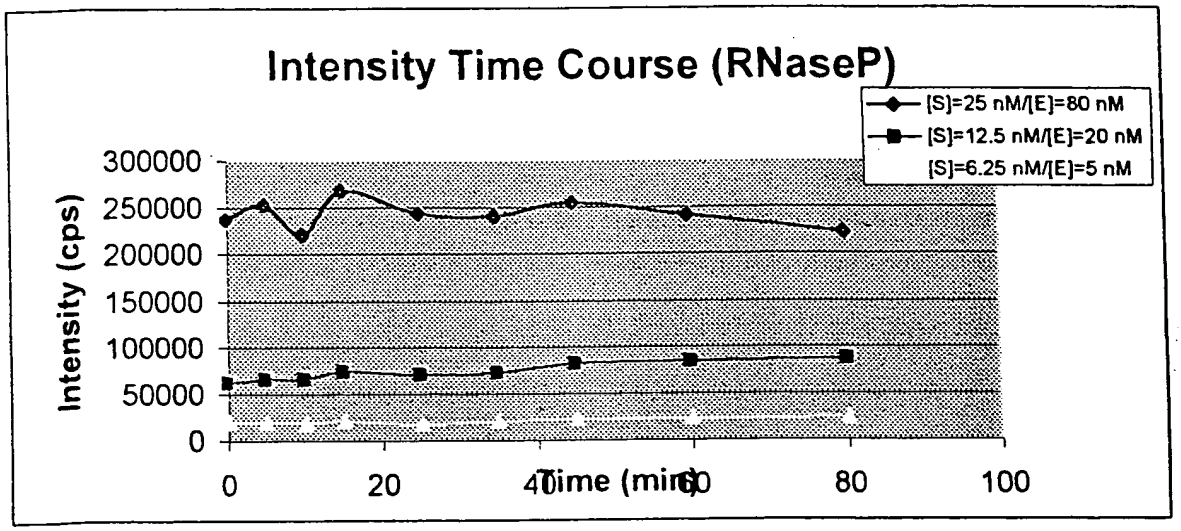
Time (min)	[S]=25 nM/[E]=80 nM	[S]=12.5 nM/[E]=20 nM	[S]=6.25 nM/[E]=5 nM
0	237731	62752	19322.5
5	253591.625	66964.25	19191.75
10	221697.875	66844.875	18739.75
15	268738.5	75114.625	20703
25	244465.625	71604.375	17584.25
35	241645.75	73591.5	19846.125
45	255650.25	83610	22297.25
60	242335.625	85644.75	22963.5
80	223405.25	88333.625	23725



The change in intensity observed on 12/17-20/99 was not reproducible in today's experiment.

There appears to be a loss of polarization over time even in the absence of enzyme. thus we need to explore additives to prevent loss of substrate(?)

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TITLE Developing RNase P Assay.

PROJECT NO.

BOOK NO.

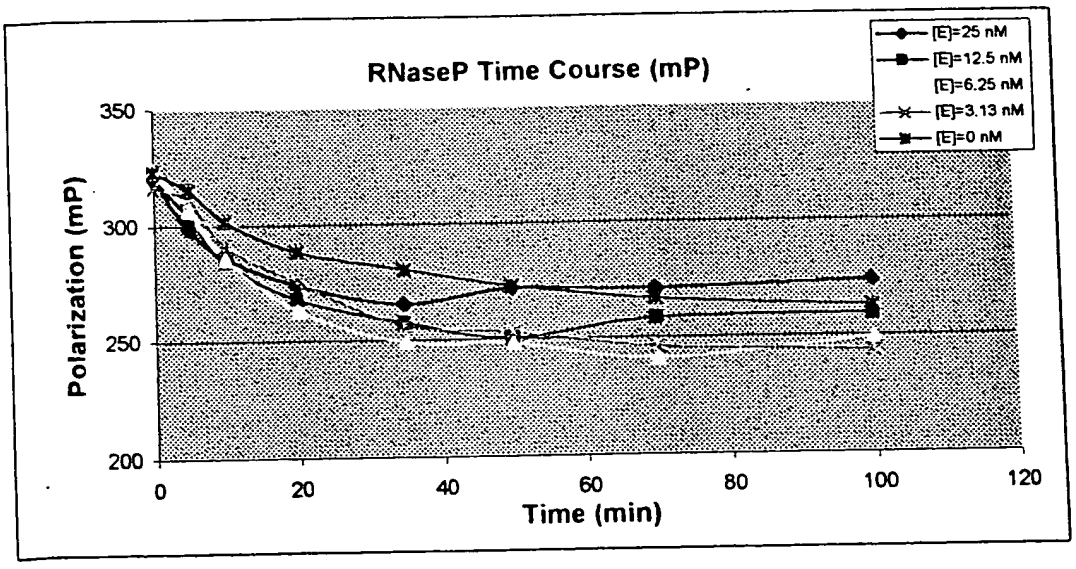
83

Work continued from Page ____

Experiment done with [S] = 25 nM and [E] = 3.1 nM, 6.25 nM, 12.5 nM, 25 nM in fresh 1X PA buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) suggested that loss of substrate polarization in the absence of enzyme (over time) was a serious problem. Gordon tried four additives to ameliorate this problem — BSA, lysozyme, PolyC and glycerol. At high concentrations some of these additives caused a decrease in intensity. It decided (based on Gordon's results) to try 10 µg/mL hen egg lysozyme, 5 µg/mL of PolyC and 2.5% glycerol in the 1X PA buffer.

Time (min)	[E]=25 nM	[E]=12.5 nM	[E]=6.25 nM	[E]=3.13 nM	[E]=0 nM
0	321.325	321.8875	323.65	316.575	323.3125
5	298.7625	304.3625	306.9875	311.925	316.025
10	286.2625	284.7	285.8875	291.5375	302.125
20	274.5125	268.3875	262.9	275.85	288.975
35	265.8625	257.9625	248.925	256.8625	280.7875
50	272.2	250.9375	248.2625	253.35	273.4
70	271.3875	258.7	241.075	246.55	267.0875
100	274.2625	259.5125	248.9	244.125	263.1125

Data is average of 8 different wells.



The additives did not particularly decrease the loss of

Work continued to Page 84

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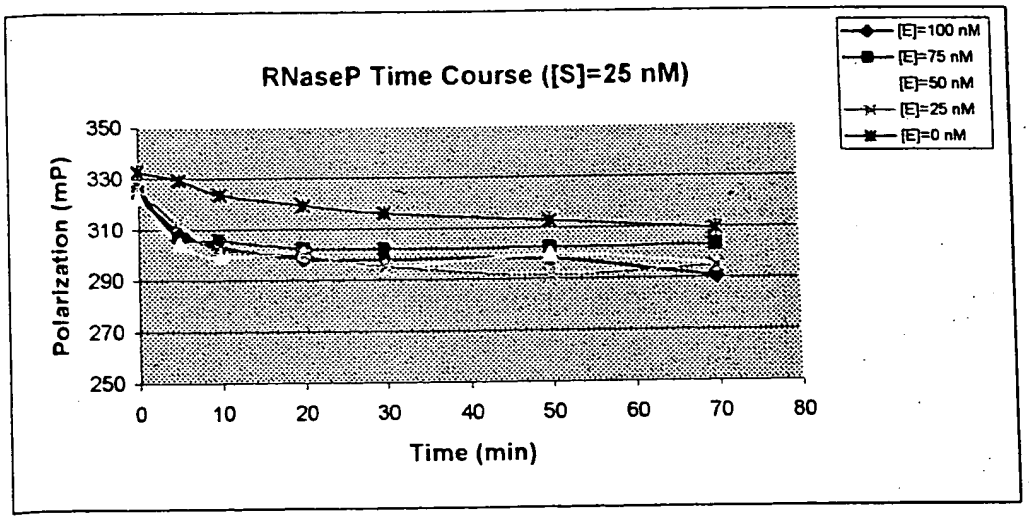
activation over time. Additionally, it was realized that the K_m for the 45 mer substrate for the enzyme is ~1 μM, and hence some of the enzyme concentrations used may be too low to get good activity of the enzyme.

The experiment was repeated (with additives) at higher enzyme concentration ([E] = 0.15 nM, 50 nM, 75 nM and 100 nM).

Time (min)	[E]=100 nM	[E]=75 nM	[E]=50 nM	[E]=25 nM	[E]=0 nM
0	323.65	326.1	322.5	326.7	333
5	309.65	307.5	304.1	312.3	329.8
10	303.7	306	299.2	302.5	323.85
20	298.7	302.7	300.4	300.2	319.7
30	298.2	302.5	294.8	295.5	316.5
50	298.1	302.6	299.6	291.9	313.2
70	290.9	303.4	295.3	295.1	309.6

Data for [E]=0 nM is average of 8 wells, for others it is average of 2 wells.



There is an initial burst of activity at all enzyme concentrations tried; which may be amenable to further optimization.

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RNase P (E.coli M1 and C5) was renatured according to standard protocol

dd H_2O = 69 µl

M1 (77 nM stock) = 3 µl

10 X PA buffer = 9 µl

Total = 81 µl

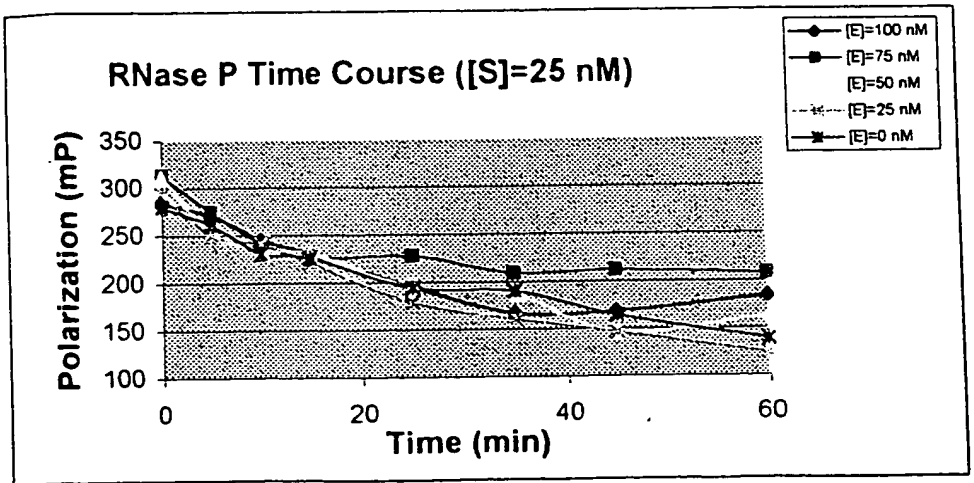
Renature successively at 65°C, 55°C and 37°C for 5 minutes each. Then 9 µl of C5 protein and 1 µl of 100 mM DTT were added and renaturation continued at 37°C for 5 minutes; the sample was then placed on ice.

The buffer 1XPA with and without additives was tried. Additives added were, 25 µg/ml HEL, 10 µg/ml PolyC and 25% glycerol.

The substrate was renatured by heating at 65°C for 5 minutes, followed by 37°C for 5 minutes.

No Additives
[S]=25 nM

Time (min)	[E]=100 nM	[E]=75 nM	[E]=50 nM	[E]=25 nM	[E]=0 nM
0	284.25	314	311.65	295.55	280.2
5	271.6	275.6	252.25	251	261.7
10	246.1	242.85	234.15	242.35	231.45
15	231	226.15	230.95	222.55	227.35
25	195.95	228.75	195.65	179.2	195.05
35	167.55	210.2	185.05	161.5	190.93
45	169.3	212.65	145.85	147.15	165.15
60	184.9	208.5	162.85	124.85	139.5



Average of two wells

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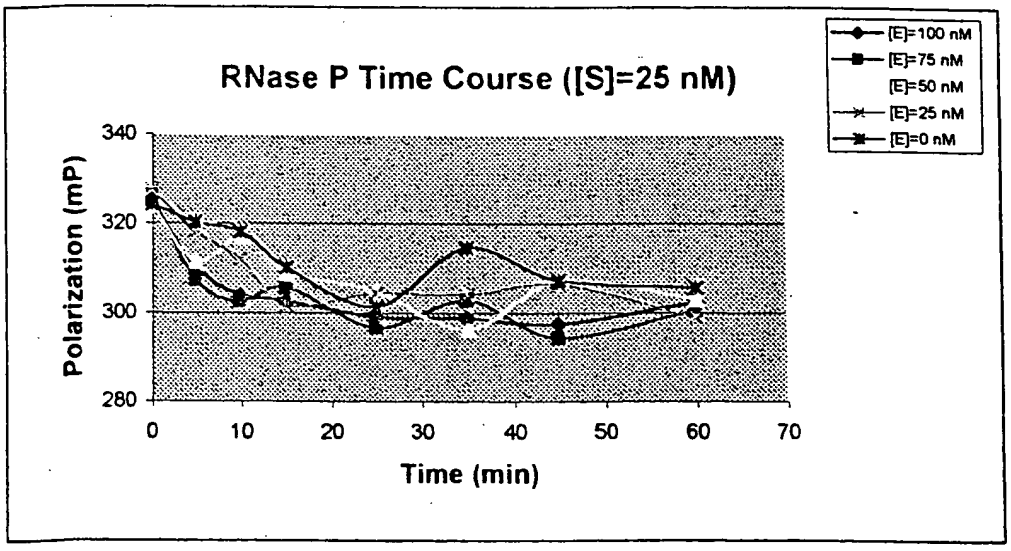
Additives
[S]=25 nM

Time (min)	[E]=100 nM	[E]=75 nM	[E]=50 nM	[E]=25 nM	[E]=0 nM
0	324.6	326.2	323.75	327.75	324.3
5	310.3	307.5	311.4	318.55	320.5
10	304.25	302.8	316.6	312.2	318.2
15	302.95	305.95	309.05	301.95	310.28
25	299.25	296.8	304.85	304.65	301.63
35	299	303	296.2	304.4	314.85
45	297.7	294.45	307.25	306.6	307.6
60	302.4	300.75	303.15	299.25	305.925



Average of two runs

The results show that in the absence of additives, there is a very significant decrease in the polarization. In the presence of additives (over one hour) the decrease in polarization is significant. ~~different~~.

The lack of change in polarization as a function of enzyme concentration may be related to the K_m of the substrate-enzyme complex. The assay is going to be repeated with higher enzyme and substrate concentrations.

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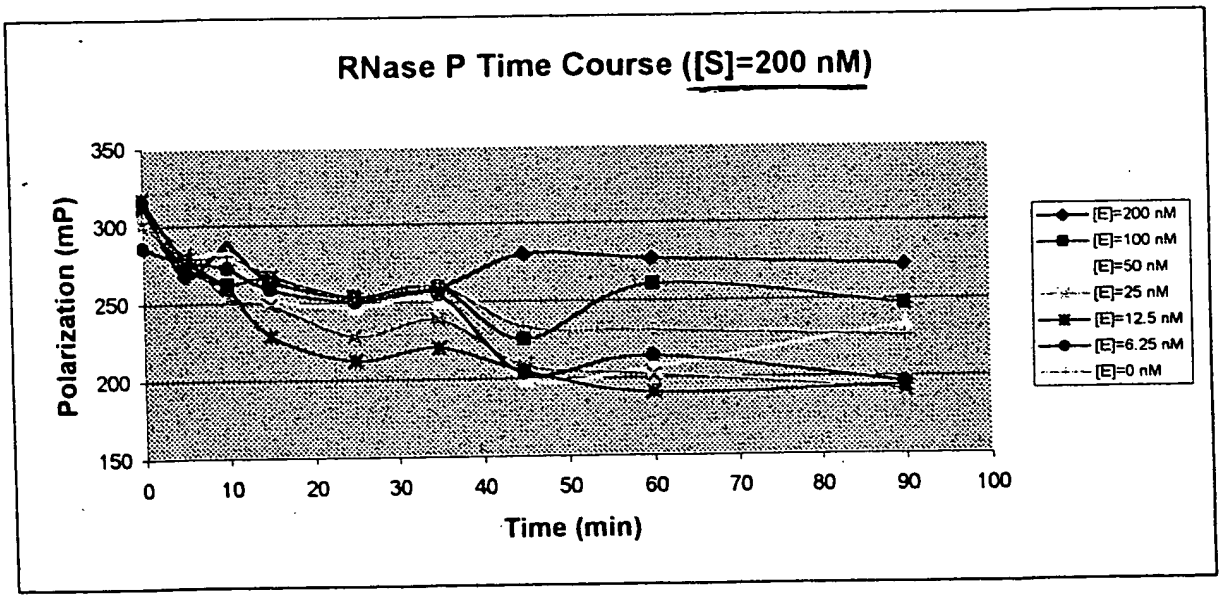
RNase P and substrate were renatured as in The 1X PA buffer contained 10 µg/mL HEL, 5 µg/mL PolyC and 2.5% glycerol.

Assay optimization

Time (min)	[E]=200 nM	[E]=100 nM	[E]=50 nM	[E]=25 nM	[E]=12.5 nM	[E]=6.25 nM	[E]=0 nM
0	317.15	309.45	305.9	301.45	318.35	286.55	306.225
5	268.5	274.8	281.45	281.65	279.5	277.3	281.075
10	286.4	262.7	281.95	254.45	260.8	273	280.625
15	264.95	266.55	252.9	249.55	229.25	259.45	269.725
25	253.7	254	246.3	228.95	212.6	250.75	252.15
35	259.325	260.275	249.6	239.25	220.925	255.1	260.9375
45	280.85	225.95	199.35	209.5	205.35	203.45	233.975
60	277.4	261.1	206.5	202.65	191.3	214.9	231.075
90	272.65	247.25	233.15	191.75	194.55	196.7	226.875



(Average of two wells)

In spite of using 200 nM substrate and higher concentrations of enzyme (50nM, 100 nM, 200 nM), there was no improvement in the change in polarization vis-a-vis control. Thus, there maybe a problem with K_m being >1 µM for this substrate.

The additives are going to be optimized for substrate concentration of ~~1 µM~~ 200 nM and the experiment repeated

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A variety of concentrations of HEL, BSA, PolyC and glycerol were tried with 200nM substrate to find conditions for least decrease in polarization with time.

Column	HEL(ug/ml)	BSA(ug/ml)	PolyC(ug/ml)	Glycerol
A	200	50	100	
B	100	25	50	
C	50	12.5	25	
D	25	6.25	12.5	
E	12.5	3.13	6.25	
F	6.25	1.56	3.13	
G	0	0	0	
H	0	0	0	

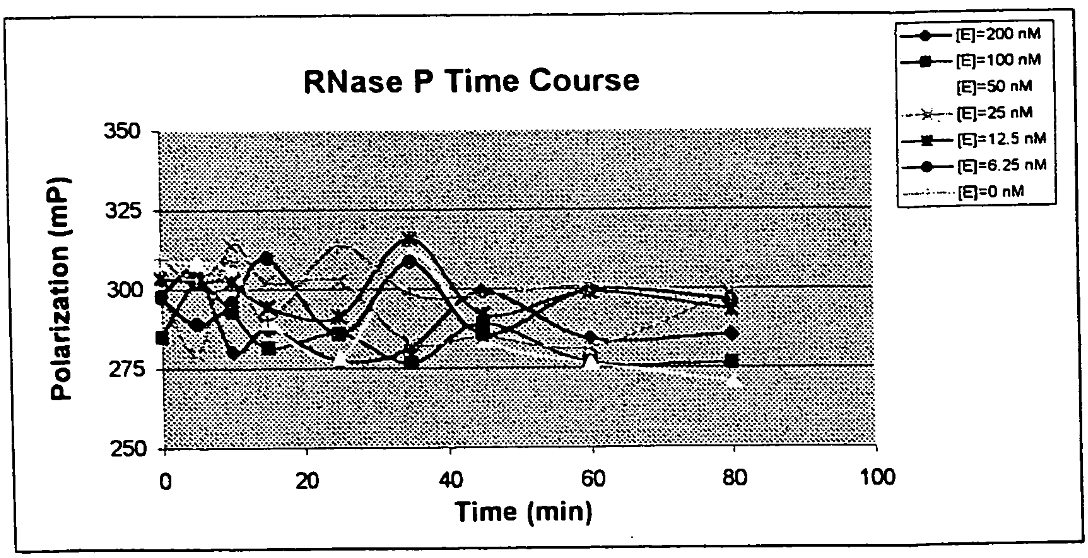
The optimal concentration of additives is:
HEL ~ 100 µg/mL
BSA ~ 25 µg/mL
PolyC ~ 50 µg/mL
Glycerol ~ 2.5%

Work continued from Page ____

RNase P and substrate were reacted as on 6/27/00.
The 1XPA buffer contained ~~10 μg/ml HEL~~ 50 μg/ml Poly C, 25 μg/ml BSA and 2.5% glycerol (HEL was not used due to Fred's gel shift experiments with RRC/RSG1.2).

[S]=200 nM

Time (min)	[E]=200 nM	[E]=100 nM	[E]=50 nM	[E]=25 nM	[E]=12.5 nM	[E]=6.25 nM	[E]=0 nM
0	298.35	285.1	308.7	300.85	303.75	297.7	309.75
5	306.05	301.9	308.3	279.95	302.5	289.45	303.575
10	280.55	293.05	305.35	314.15	302.5	296.15	309.1
15	287.85	281.65	289.85	302.6	294.6	309.95	290.7
25	277.5	286.25	278.5	302.75	291.7	285.95	313.875
35	281.8	276.8	311.1	283.25	316.1	308.85	298.65
45	299.3	289.4	283.85	285.2	292.15	285.85	298.275
60	284.3	276.85	276.5	281.85	299.05	299.7	301.075
80	285.55	276.75	270.45	298.75	293.55	296.45	297.2



(Average of two wells)

The addition of additives certainly reduced the ~~decrease~~ loss of polarization. However, there was no change in polarization as a function of enzyme concentration.

We need to consider a substrate with larger Km for the enzyme.

Work continued to Page ____

SIGNATURE K. Anil Xavier DATE

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To test the cleavage reaction was indeed occuring, we decided to setup the enzyme reaction in a microfuge tube with and without additives.

In each tube final concentration of enzyme is 400 nM and substrate (pAT45) is 2μM. The enzyme was renatured according to standard protocol. Stock concentrations of enzyme and substrate were 8.6 mM and 76 μM respectively.

Tube #	Enzyme (μl)	2x PA (μl)	pAT45 (μl)	dd H_2O (μl)	Additive
1	10	5	0.6	4.4	—
2	10	5	0.6	4.0	0.4μl
3	10	5	0.6	4.0	0.4μl
4	10	5	0.6	4.3	0.1μl
5	10	5	0.6	3.2	0.4+0.4 (all three)
6	0	5	0.6	14.4	—

The stock concentrations of HEL, Poly C and BSA are 20 mg/ml, 10 mg/ml and 40 mg/ml respectively; hence the final concentrations in respective tubes are 200 μg/ml, 200 μg/ml and 200 μg/ml.

The reaction was done for 45 minutes at room temperature. 20 mM EDTA (final concentration) was added to quench the reaction.

10 μl from each tube was diluted 10X and the polarization determined on the LJL analyst (this was done after adding 15 μl of formamide to each tube and heating the contents for 7 minutes). 20 μl from the tube was loaded on a 10% denaturing gel.

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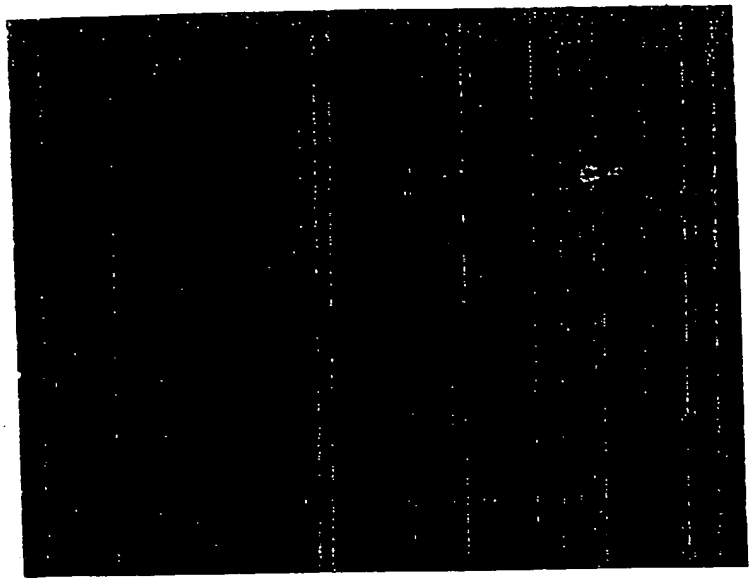
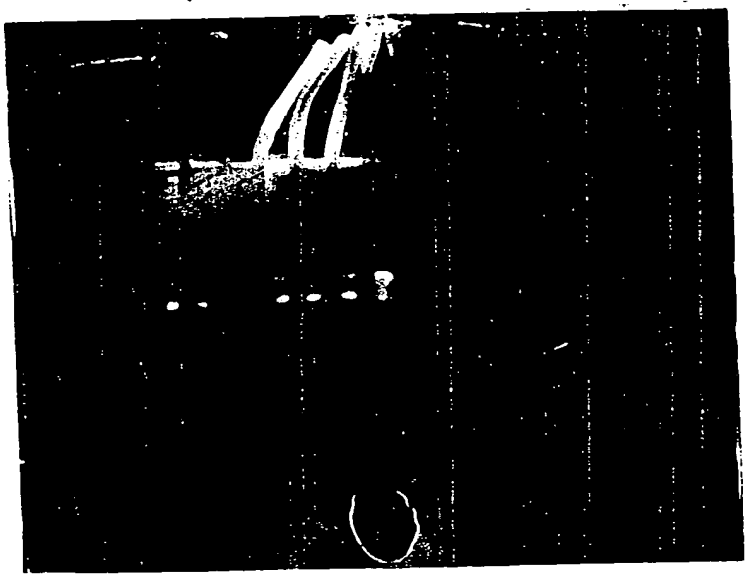


The gel photographed with UV lighting, clearly showing two distinct bands, corresponding in all likelihood to the 45mer and the 10mer.



The gel photographed after staining with ethidium bromide.

Description	Reading 1	Reading 2	Reading 3	Average
Buffer	630.1	598.8	623.6	
Buffer	632.9	637.1	614.8	
Tube #1	152.6	160.4	163.1	158.7
Tube #2	193.3	160.6	180.4	178.1
Tube #3	244.1	241.2	208.5	231.2666667
Tube #4	212.4	155.4	148.7	172.1666667
Tube #5	252.3	240.8	239	244.0333333
Tube #6	260.4	225.1	239.3	241.6

Except in the presence of PolyC, there is a clear and significant decrease in polarization on ~~addi~~ enzyme cleavage.

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The enzyme was reactivated in 1X PA according to standard protocol. The substrate was warmed at 37°C for 5 minutes. Stock solutions of the enzyme and the substrate were 300 nM and 70 nM respectively.

Tube #	Enzyme (µl)	2x PA (µl)	pATAS (µl)	dH2O (µl)	Additives
1	20	10	1.2	8.8	—
2	20	10	1.2	8.0	0.3 µl (HEL)
3	20	10	1.2	8.0	0.3 µl (PolyC) &
4	20	10	1.2	8.0	0.2 µl (BSA) &
5	20	10	1.2	6.4	0.3 µl HEL 0.2 (All three)
6	20	10	1.2	28.3	—

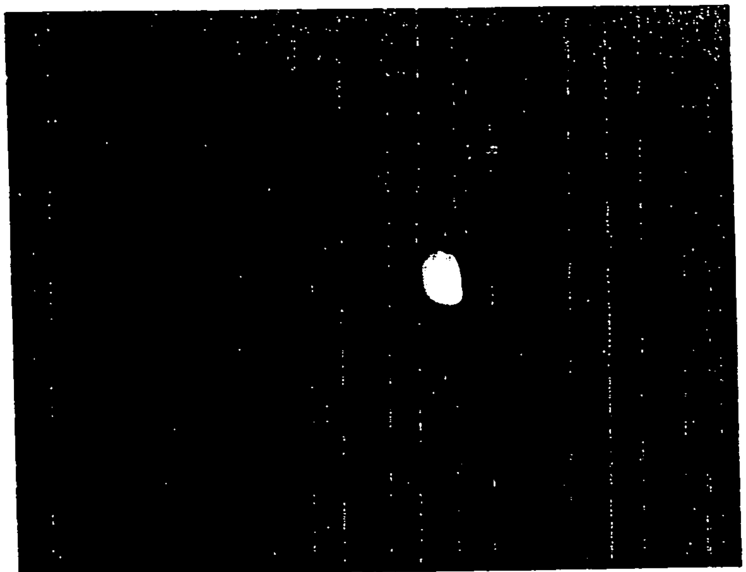
The stock concentrations of HEL, PolyC and BSA were 10 mg/ml, 10 mg/ml and 40 mg/ml respectively; hence the final concentrations in respective tubes are 200 µg/ml, 200 µg/ml and 200 µg/ml.

The reaction was done for 45 minutes at room temperature. 20 mM EDTA (final concentration) was added to quench the reaction.

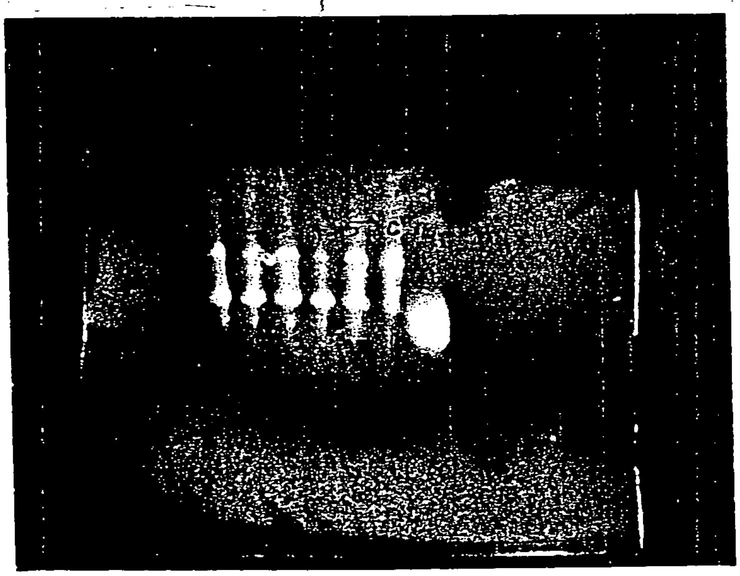
10 µl from each tube was diluted 10-fold and polarization determined on the LTL analyzer.

Description	W1R1	W2R1	W1R2	W2R2	W1R3	W2R3	Average
Buffer	701	666.7	701.5	670	689.1	690.1	686.40
Tube#1	159.1	124.4	155.9	101	137.4	94.3	128.68
Tube#2	137.8	152.4	173.4	135.6	151.6	170.6	153.57
Tube#3	111.5	176.8	141.5	139.7	100.2	127.5	132.87
Tube#4	222.8	259.6	225.7	254.1	247.4	264.5	245.68
Tube#5	253.3	217.1	201.6	221.4	230.4	228.1	225.32
Tube#6	278	266.2	274.8	244.4	238.8	287.2	264.90
10mer	166.8	176.7	127.8	137.9	161.9	161.5	155.43

Work continued from Page 92



1/17/00 Additive Assay (unstained)



Thus after a 45 minute incubation the substrate is significantly cleaved and in the presence of certain additives there is a corresponding decrease in the measured polarization.

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94 **TITLE** Testing the Bipartite Substrate **PROJECT NO.**

Work continued from Page ____ **BOOK NO.**

An experiment was set-up to test the cleavability of the bipartite substrate (10mer Cy3 labeled + 5mer). The enzyme concentration used was 3.1 μM, 6.25 nM, 12.5 nM, 25 nM, 50 nM and 100 nM. The substrate concentration was kept fixed at 100 nM (reactions at 65°C for 5 minutes and slowed cooled). Additives used in the 1X PA buffer were HEL @ 100 μg/mL BSA @ 50 μg/mL and HEL @ 100 μg/mL + BSA @ 50 μg/mL. The data for a time course over one hour was inadvertently lost. The general observations on following the experiment were:

- presence of BSA was detrimental to measurement of fluorescence polarization
- HEL was useful in reducing the loss of fluorescence over time presumably due to loss of substrate
- there was a range of enzyme concentrations (3.1 μM, 6.25 nM and 12.5 nM) were the decreased polarization approached that for the 10mer Cy3 labeled product.
- at high enzyme concentrations in significant decrease in polarization over time was observed.

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Work continued to Page ____

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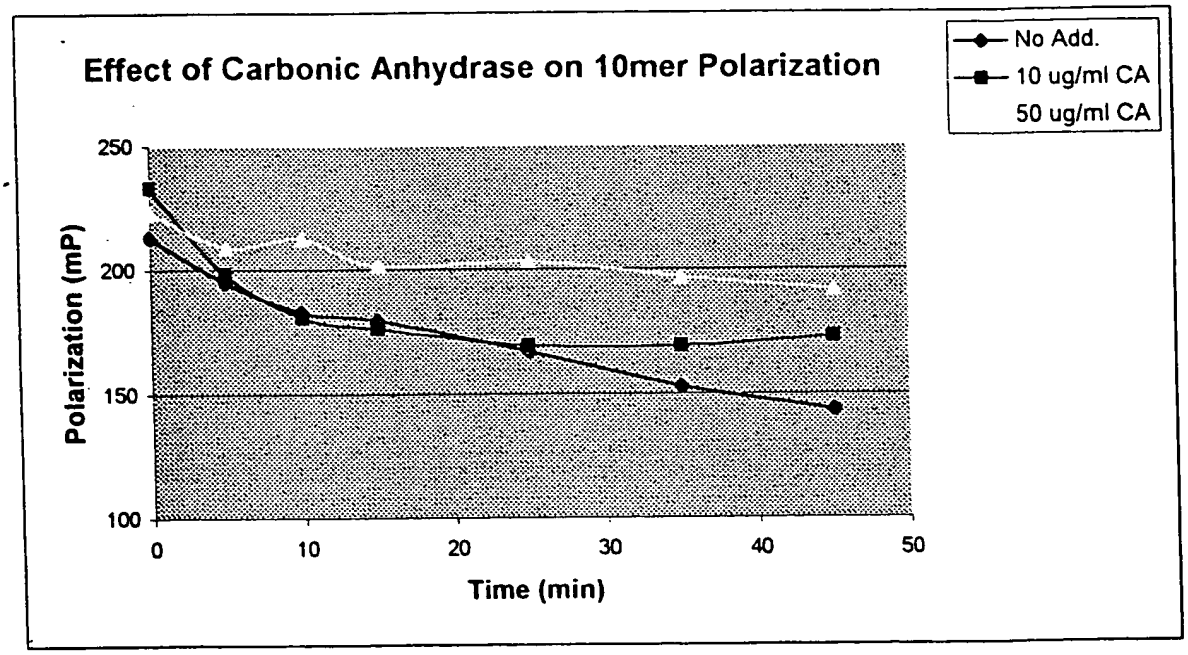
The Cy3 labeled 10 nucleotide leader sequence was used to test various carbonic anhydrase concentrations to investigate if carbonic anhydrase prevents loss of fluorescence polarization over time.

Time (min)	No Add.	10 ug/ml CA	50 ug/ml CA
0	213.5875	233.45	223.025
5	195.125	198.9	209.25
10	183.225	181.3	212.625
15	180	176.575	201.35
25	167.3625	169.8375	203.0625
35	152.9625	169.6	196.625
45	143.7125	173.1875	191.8375



Subsequent experiments by Gordon suggested that carbonic anhydrase concentrations in the 10-35 ng/ml does not interfere with RNase P cleavage reaction.

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A buffer containing 25 µg/ml carbonic anhydrase, 25 µg/ml PolyC, 25 µg/ml hen egg lysozyme and 2% glycerol was tried at three enzyme concentrations (1 nM, 5 nM and 10 nM). As controls, the substrate without enzyme and 50 nM 10mer labeled with Cy3 were used. The substrate was at final concentration of 50 nM.

The substrate components were mixed as follows:

Labeled 26mer (~152 µM) = 1.7 µl
In vitro transcribed 57mer (~256 µM) = 1.3 µl (~20% excess over 26mer)
10X PA buffer = 25 µl
dd H_2O = 222 µl
Total = 250 µl

Final concentration of substrate would be ~1 µM. The substrate was heated at 65°C for 5 minutes and then warmed at 53°C and 37°C for 5 minutes each.

The assay did not work, there was little change in polarization over 1½ hours; which in retrospect was probably because 10X PA buffer was inadvertently left out of the buffer!

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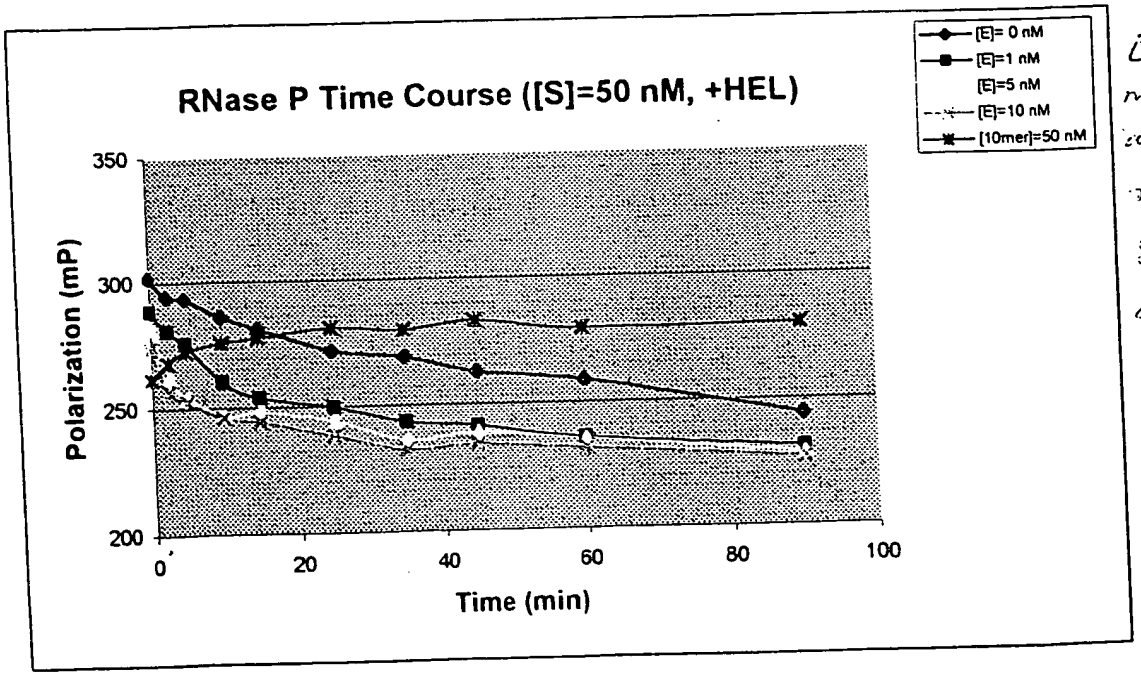
The buffer used was 1XPA, 2% glycerol, 25 μg/ml HEL, 25 μg/ml carbonic anhydrase, 50 μg/ml PolyC, 10 mM DTT. The bipartite substrate was renatured as in vitro except for two changes the final concentration of substrate during annealing was ~5 μM and 100% excess of 59 mer over 26mer was used. Note: Buffer with and without HEL was tried with black polystyrene plates.

[S]=50 nM, +HEL

Time (min)	[E]= 0 nM	[E]=1 nM	[E]=5 nM	[E]=10 nM	[10mer]=50 nM
0	302.32	288.975	282.1	274.1125	262.0125
2.5	294.56	281.0875	262.45	257.0375	268.2375
5	293.88	275.875	254.0375	252.75	272.775
10	286.54	260.5875	246.6125	246.35	276.075
15	281.5	254.0875	248.125	244.5125	277.85
25	272.38	249.925	244.05	238.475	281.625
35	269.34	243.625	235.95	232.7625	280.4
45	262.98	241.6125	238.6375	234.3875	283.6
60	259.16	236.1625	235.525	232.125	279.7
90	244.28	231.2875	228.35	227.2375	280.8



Each measurement is an average of readings from 3 different wells.

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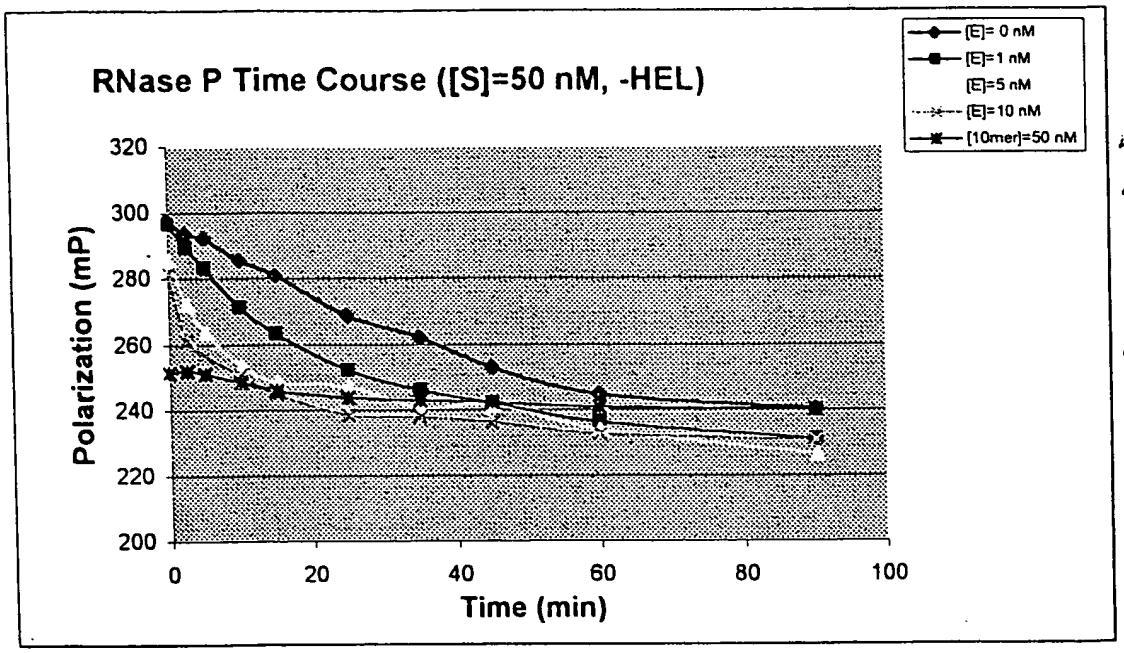
[S]=50 nM, -HEL

Time (min)	[E]= 0 nM	[E]=1 nM	[E]=5 nM	[E]=10 nM	[10mer]=50 nM
0	297.9375	296.8875	285.4375	282	251.6
2.5	293.975	289.925	272.225	261.5375	252.2625
5	292.6875	283.525	263.65	257.0625	251.2875
10	285.9	271.6625	253.15	251.0625	248.7125
15	281.275	263.55	248.5375	245.1875	246.2375
25	269.125	252.375	247	238.7625	244.125
35	262.4125	246.475	240.5125	238.1375	243.1625
45	253.2875	242.2375	241.575	236.5375	242.4625
60	245.05	236.5625	233.925	233	241.125
90	240.5125	231.3875	226.5	231.05	240.2375



Each measurement is an average of readings from 3 different wells.

The presence or absence of hen egg lysozyme does not have much of a difference.

There is definitely an enzyme concentration dependent decrease in polarization with time, indicating cleavage of substrate by RNase P. The decrease is sufficient to base an assay around, however over time the wells with no enzyme also has a significant decrease in polarization. As opposed to this, the Cy5 labeled 10mer has a fairly constant polarization.

Work continued to Page

SIGNATURE K Anita Xavier DATE

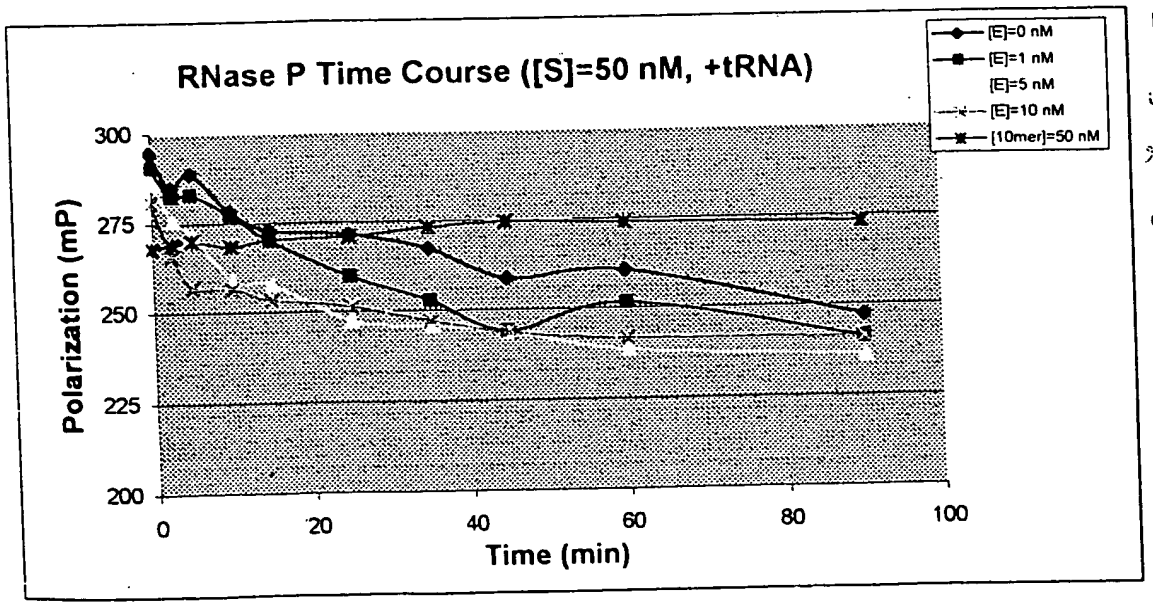
DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

The buffer used was 1XPA, 2% glycerol, 25 µg/mL HEL ~~50~~ 25 µg/mL carbonic anhydrase, 50 µg/mL PolyC, 10 mM DTT and 25 µg/mL tRNA. The bipartite substrate was annealed according to protocol of a/24/00 except for two changes: the final concentration of substrate during annealing was 0.5 µM and 100% excess of 59mer over 26mer was used.

Note: Buffer with and without tRNA was tried with white polypropylene plates.

[S]=50 nM, +tRNA

Time (min)	[E]=0 nM	[E]=1 nM	[E]=5 nM	[E]=10 nM	[10mer]=50 nM
0	294.7625	290.7375	286.5375	281.0125	268.3125
2.5	284.9625	282.7	275.7125	265.9375	269.0875
5	289.0375	283.1375	271.8875	257.425	270.1875
10	278.275	277.375	258.8375	256.7625	268.65
15	273.075	270.3375	257.7875	253.6875	270.375
25	271.7375	260.1	247.225	251.5875	271.0875
35	267.625	253.0125	245.5375	247.3625	273.45
45	258.9	244.3375	243.5875	243.95	274.675
60	260.825	251.7625	238.2625	242.0125	274.1125
90	247.275	241.3	236.1	241.4125	273.55



Each measurement is an average of readings from 8 different wells.

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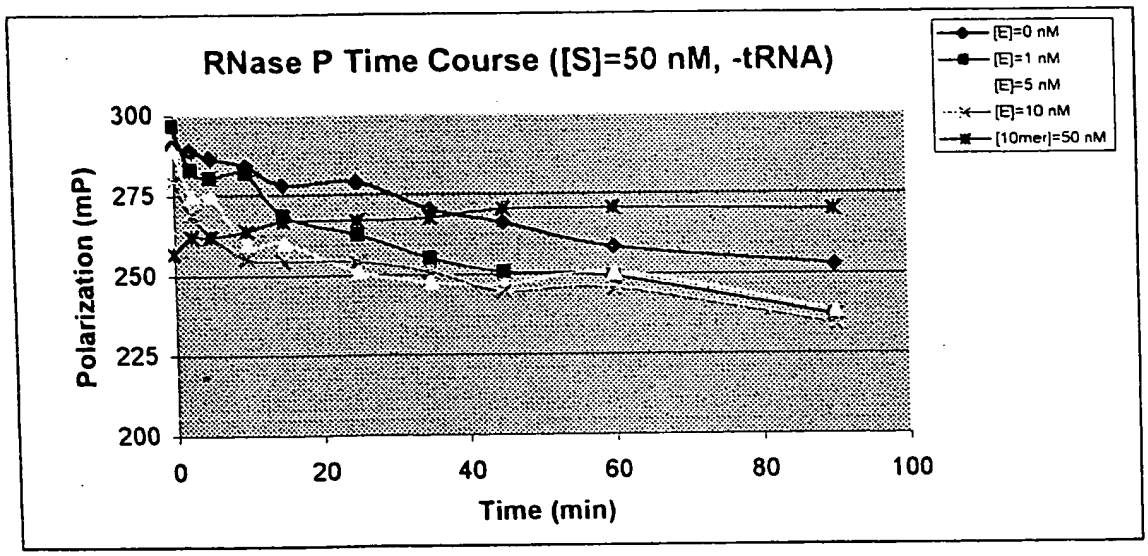
[S]=50 nM, -tRNA

Time (min)	[E]=0 nM	[E]=1 nM	[E]=5 nM	[E]=10 nM	[10mer]=50 nM
0	290.575	296.975	289.1875	279.7125	257.125
2.5	289.4625	283.4	274.65	269.5625	262.25
5	287.05	280.7125	274.75	262.8875	262.225
10	284.575	282.1125	259.775	255.4625	264.1
15	278.5	268.9375	260.175	254.7625	267.0375
25	279.3375	263	250.95	254.575	267.425
35	270.8125	255.5125	247.55	251.0375	268.225
45	266.525	251.1125	246.7125	245.125	270.8
60	258.975	249.4125	250.5875	245.9125	270.975
90	253.1125	237.2875	238.5	234.525	270.3125



Each measurement is an average of readings from 4 different wells

The presence of absence of tRNA or the use of polypropylene plate does not result in drastic difference in the observed change in polarization with time.

The addition of tRNA most importantly does not affect the activity of the RNase P enzyme, on the other hand the tRNA does not prevent loss of substrate signal over time.

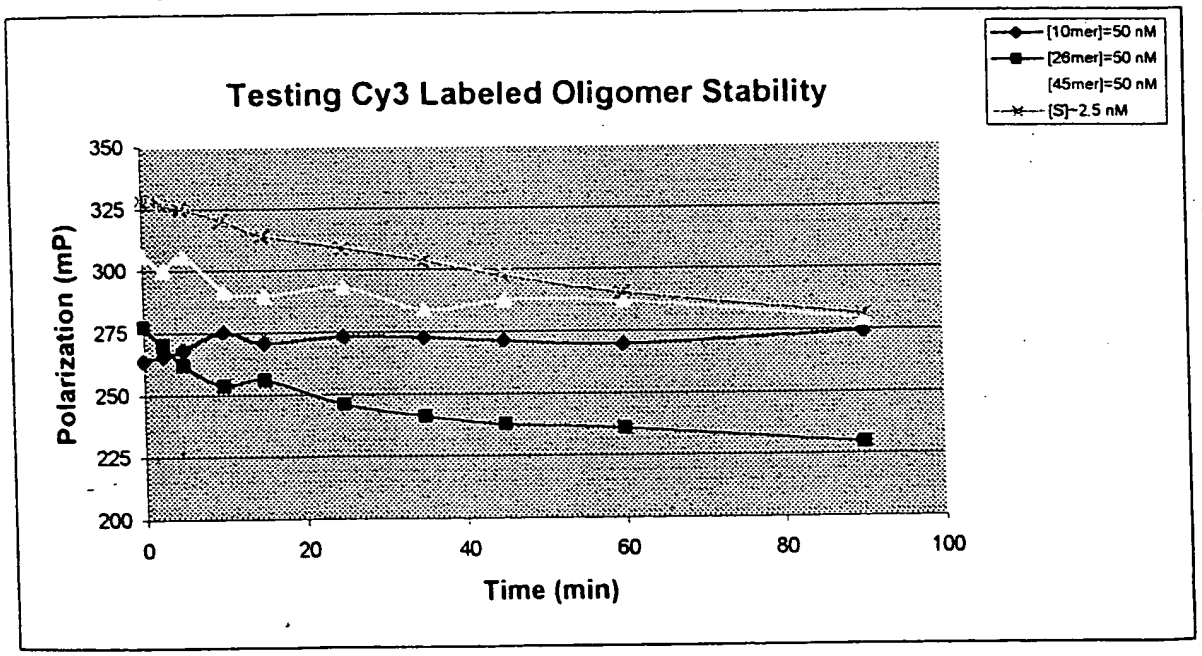
One possibility is the substrate is not properly annealed or is dissociating over time either due to dilution in the assay or due to weak annealing.

SIGNATURE K Anish Xavier DATE

DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

Time course for the Cy3 labeled 10mer, 26mer, ptA45 (45mer) and the bipartite substrate (26mer + 5'mer) in buffer from 1/26/06 containing both AHL and tRNA was done. The final concentration of each RNA (except the bipartite substrate) was ~5 nM, there was not sufficient 5'mer for annealing therefore the concentration of the 5'mer was ~2.5 nM.

Time (min)	[10mer]=50 nM	[26mer]=50 nM	[45mer]=50 nM	[S]~2.5 nM
0	263.9625	277.7125	306.1	329.1125
2.5	265.8875	270.4375	299.825	326.9875
5	268.3	262.3375	305.5375	325.125
10	275.6375	254.075	291.9125	320.8125
15	271.075	256.45	289.85	314.55
25	273.375	246.1875	292.7875	309.3875
35	272.9	241.4125	283.8125	304.35
45	271.5375	237.9375	287.4125	298.1
60	270.2125	236.0875	286.85	290.65
90	274.3625	230.1125	279.5125	281.6625



Each measurement is an average of 8 different wells

Both the 10mer and the 45mer are fairly stable over time. The behavior of the 26mer and the bipartite substrate are similar, while the higher polarization of the bipartite substrate suggests that it is annealed.

SIGNATURE K Anish Xavier DATE /

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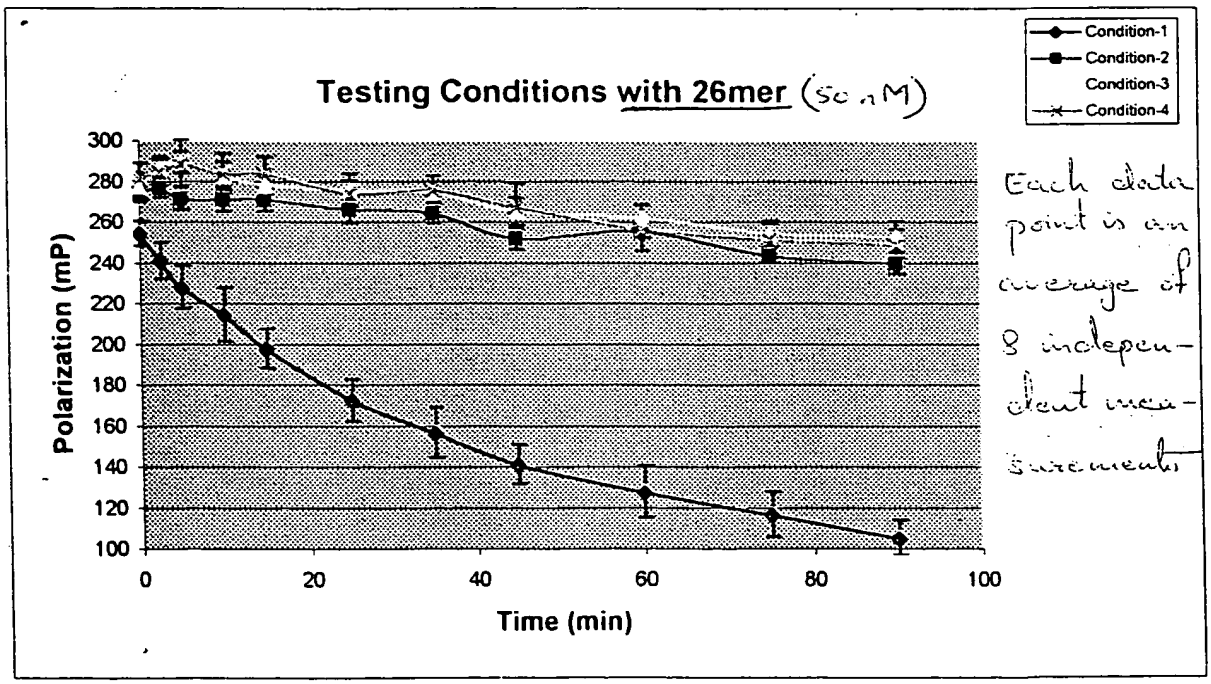
Condition-1 1X PA + 2% glycerol + 10 mM DTT

Condition-2 1X PA + 2% glycerol + 10 mM DTT + 25 µg/mL carbonic anhydrase

Condition-3 : Condition-2 + 50 µg/mL PolyC + 25 µg/mL tRNA (Sigma)

Condition-4 : Condition-3 + 0.03% NP-40.

Time (min)	Condition-1	Condition-2	Condition-3	Condition-4	SD-1	SD-2	SD-3	SD-4
0	254.5	274.9375	277.7125	281.8	6.1	5.1	6.8	7.3
2.5	241.025	276.9	286.3625	284.8375	9.04	4.99	4.39	7.16
5	227.975	271.55	289.2375	288.925	10.6	5.48	5.3	11.3
10	214.6125	271.2875	282.7625	284	13.3	6.17	6.87	9.66
15	197.8875	271.2875	278.375	284.3375	9.84	5.97	4.82	7.84
25	172.625	266.5	275.7625	274.3625	10.3	6.82	8.25	5.95
35	156.6625	264.6625	274.2875	276.2125	12.4	5.41	5.11	6.4
45	141.225	252.45	264.8875	267.675	9.6	6.03	6.86	11.2
60	127.75	256	261.8375	256.9125	12.8	3.78	6.81	11.2
75	116.725	243.9875	254.4125	251.7125	11	4.06	6.17	7.25
90	105.2625	240.2625	252.8	249.375	8.53	5.75	7.53	6.96



Each data point is an average of 8 independent measurements

Certainly a non-specific protein (carbonic anhydrase) is required, else the condition produce identical results.

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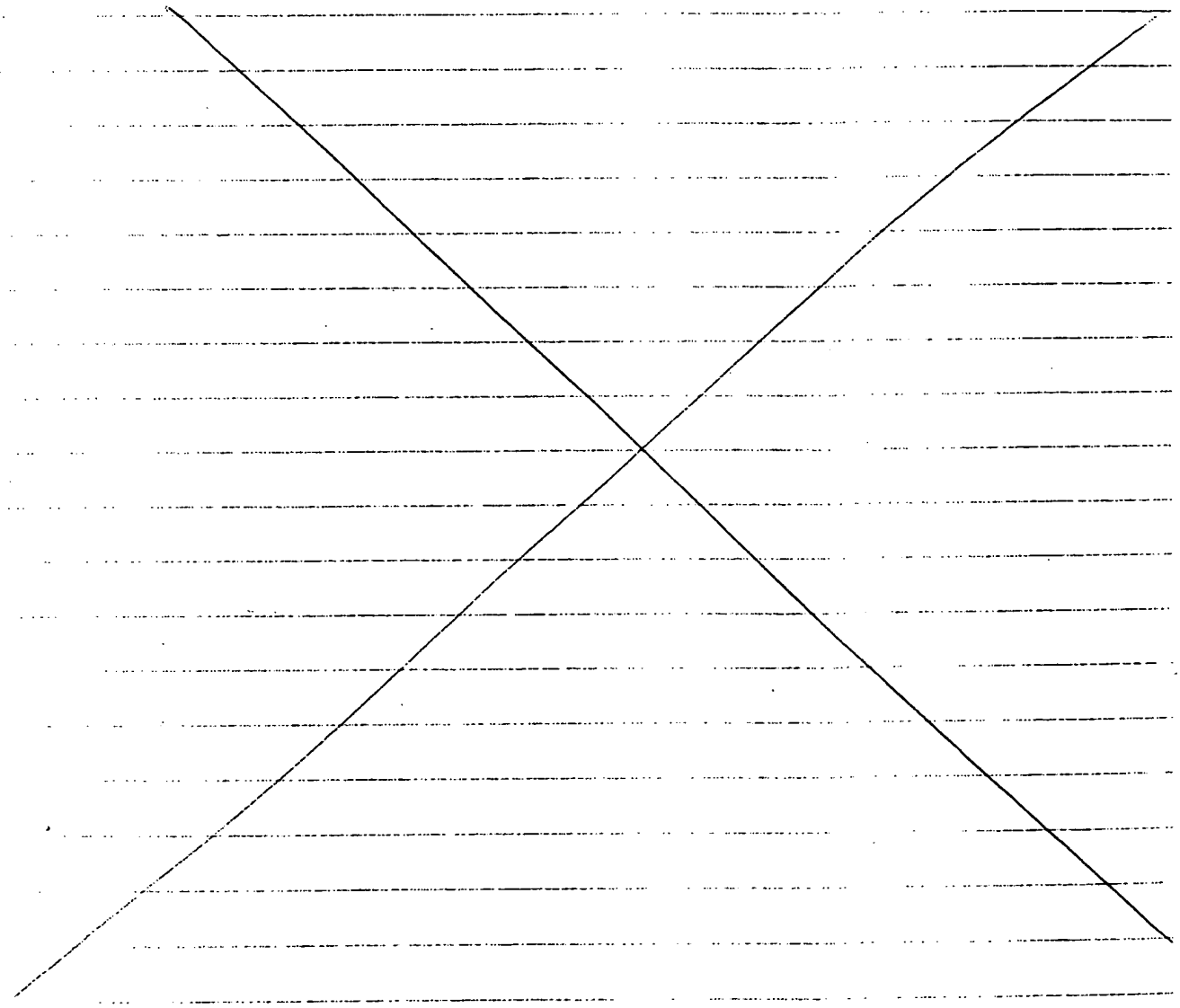
Work continued from Page ____

Experiment was done with 40 nM annealed bipartite substr and renatured RNase P at concentrations of 4 nM, 2 nM, 1 nM, 0 nM, 0.5 nM and 0.25 nM (final concentration) to explore what is the lowest enz concentration at which there is no decrease in observed cleavage over about 30 minutes.

The experiment essentially showed that ~5 nM RNase P is required for the largest change in measured polar ization with $ptRNA^{Gln}$



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Paul Eder decided on two pre-tRNA ~~subs~~ substrates from Staph aeurus, ptRNA^{Thr} and ptRNA^{Leu}. The substrate will be synthesized in two fragments for ease of labeling. Using Zuker's program the 24 nt fragment of ptRNA^{Thr} showed the least likelihood of having any structure and therefore ptRNA^{Thr} was chosen to be the substrate with the most likelihood of success.

ptRNA^{Thr} (24mer): uuu cuu AgC Cgg CCu AgC UCA AUU

ptRNA^{Leu} (24mer): uuA uAg UgC Cgg ggU ggC ggA ACU

Different approaches for developing an RNase P assay:

1) Change in FP

Use bipartite substrate 5'-labeled with Cy3 or TAMRA expecting a change in measured polarization on substrate cleavage.

2) 5' labeling of (GMP modified) 5'-thiol 85mer

Incorporate thiol at 5' position during in vitro transcription. Using iodoacetamide derivative of TAMRA to label substrate; expecting a change in measured polarization on substrate cleavage.

3) IGEN Technology

5' label 24mer with ruthenium chelate and 3' label with biotin. Then use IGEN instrumentation for detection.

4) Time resolved fluorescence assay using streptavidin plates

5' ~~and~~ label 24 mer with biotin and label the anti-codon loop with biotin. Adsorb 5'mer to streptavidin plate, then anneal the whole ptRNA, cleave and TRF detection.

Work continued to Page ____

SIGNATURE K Anil Kumar DATE

DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

Experiment was set-up such that either 1X PA or 10 mM MOPS (pH=7.5), 100 mM NH_4Cl & 10 mM $MgCl_2$ was used as base buffer. The additives used were 25 µg/mL carbonic anhydrase, 50 µg/ml PolyC, 25 µg/mL tRNA, 10 mM DTT and 2% glycerol.

Also, after 20 minutes of cleavage EDTA was added to a final concentration of 50 mM and the polarization measured for a further 20 minutes.

This experiment was performed as Gordon had observed in preliminary experiments >50 mP changes in MOPS buffer on addition of EDTA.

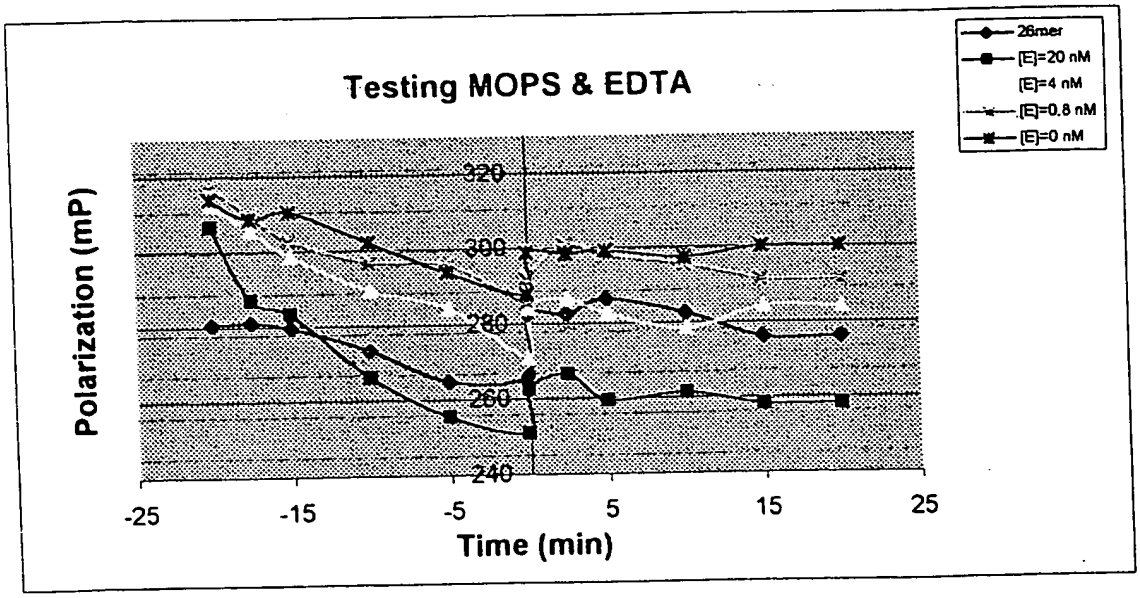
26mer @ 50 nM concentration was used as control to understand the effect of adding EDTA. The experiment was performed at [E] = 0, 0.8 nM, 4 nM and 20 nM with annealed biparti substrate at 50 nM.

Control

Time (min)	26mer	[E]=20 nM	[E]=4 nM	[E]=0.8 nM	[E]=0 nM
-20	283.55	303.075	313.575	315.375	315.725
-17.5	293.4	289	306.575	317.55	317
-15	281.85	279.675	300.25	305.875	310.6
-10	282.225	262.375	290.6	303.825	305.8
-5	279.325	259.2	282.375	300.825	306.35
0	276.35	258.125	275.85	296.75	298.8
0	293.575	266.3	281.175	303.2	307.6
2.5	288.7	271.25	285.1	303.325	308.5
5	293.125	267.875	285.15	304.55	310.65
10	287.075	268.875	287.85	300.8	310.35
15	290.35	266.9	283.925	299.75	310.8
20	290.575	265.95	287.3	301.025	307.4

MOPS

Time (min)	26mer	[E]=20 nM	[E]=4 nM	[E]=0.8 nM	[E]=0 nM
-20	280.825	306.875	312.475	317.375	314.1
-17.5	281.375	287.275	305.525	310.025	308.725
-15	279.875	283.475	298.775	303	310.725
-10	273.65	266.625	289.4	297.075	302.4
-5	265.25	255.75	284.425	296.075	294.15
0	266.475	251.15	270.925	285.7	288.15
0	282.7	263	284.3	292.275	299.125
2.5	282.9	266.925	286.6	301.275	298.9
5	286.75	259.875	282.6	298.175	299.6
10	282.825	261.75	278.475	295.625	297.75
15	276.35	258.625	284.05	291.575	300.425
20	276.35	258.625	284.05	291.575	300.425

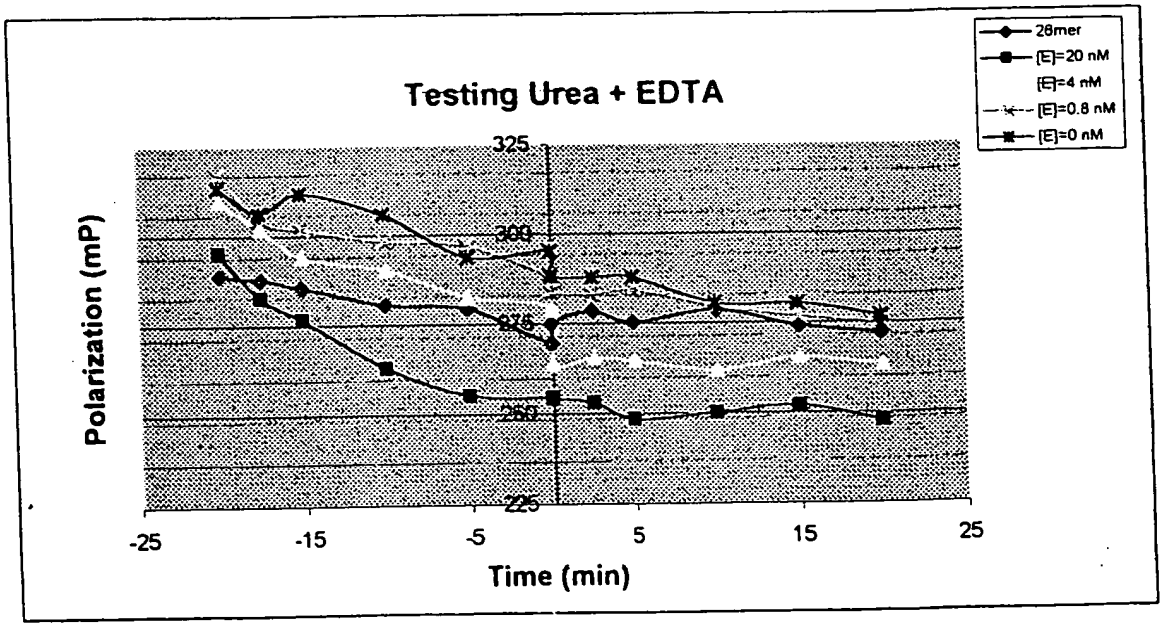


* In the first 20 minutes of enzyme cleavage there is no significant difference between using Tris or MOPS as the buffer.
* On addition of EDTA the measured polarization increases and the signal is stable for the next 20 minutes. However, EDTA does not cause an increase in the net difference in polarization when enzyme is present vs. when enzyme is absent.

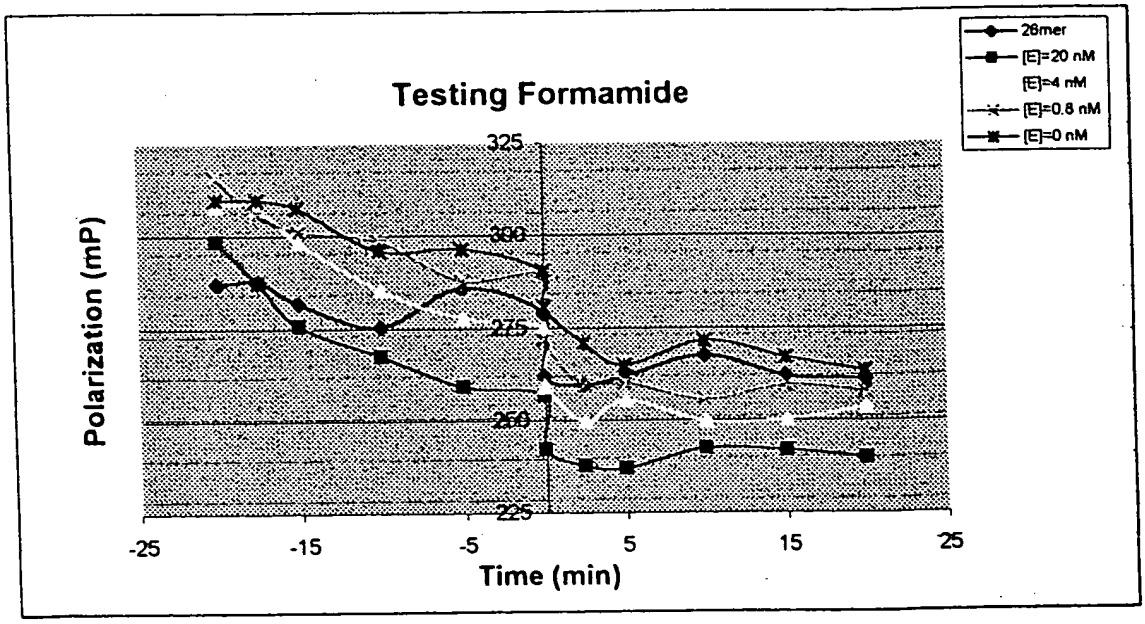
Work continued from Page ____

Experiment similar that performed on ____ was done using 1.6M urea + 50 mM EDTA (final concentration) or 20% formamide (final concentration) as the denaturant. [E] tested where 0 nM, 0.8 nM, 4 nM and 20 nM, and a fixed substrate concentration of 50 nM.

Urea+EDTA Time (min)	26mer	[E]=20 nM	[E]=4 nM	[E]=0.8 nM	[E]=0 nM
-20	289.1	295.375	309.6	316.925	313.75
-17.5	288.15	282.9	301.825	304.575	306.525
-15	285.75	276.8	293.975	301.65	312.025
-10	280.65	263.125	290.225	298.35	306
-5	279.725	255.4	282.6	297.025	293.95
0	269.775	254.75	279.175	288.025	295.6
0	275.35	254.45	263.65	283.9	288.8
2.5	278.675	253.375	265.275	283.225	288.275
5	275.475	248.6	264.5	284.5	288.175
10	278.825	250.2	261.825	279.575	280.675
15	274.375	252.2	265.225	277	280.5
20	272.275	248	263.125	274.75	277.075



Formamide Time (min)	26mer	[E]=20 nM	[E]=4 nM	[E]=0.8 nM	[E]=0 nM
-20	287.275	298.9	308.5	316.125	310.175
-17.5	287.9	287.525	307.625	306.25	310.15
-15	282.075	275.95	298.35	301.55	307.8
-10	275.65	267.75	285.275	298.75	296.25
-5	286.25	259.525	277.725	288.025	296.775
0	279.05	257.125	274.85	289.6	290.9
0	262.275	242.55	259.55	272.25	281.175
2.5	259.775	237.95	249.55	259.85	271.25
5	262.15	237.275	255.275	260.55	265.275
10	267.875	242.625	249.525	255.825	271.75
15	262.3	242.125	250.1	259.75	267.25
20	261.4	240.15	253.425	257.95	263.6



Both urea and formamide decreased the measure polarization (formamide more so), however they did not significantly affect the difference in polarization when enzyme is present versus when enzyme is absent.

RNase P (Staph) was renatured according to standard protocol. The stock solutions of Staph RNA and Staph Protein were ~20 μM and ~100 μM respectively.

RNA M1 = 2 μl

5x modified PA = 8 μl

dd H_2O = 24 μl

Renature for 5 minutes at 65°C, 55°C and 37°C respectively. Then add:

Protein cs = 2 μl

DTT = 4 μl

Continue renaturation at 37°C for 5 more minutes. Final concentration of renatured RNase P is ~1 μM.

Substrates were annealed at 65°C, 55°C and 37°C for 5 minutes each in order to constitute the full-length ptRNA.

Stock solution of pThr (24mer)/pLeu (24mer) ~1.8 μg/μl = 225 μM

Stock solution of pThr (59mer)/pLeu (59mer) ~1 μg/μl = 50 μM

To get a final concentration of either $ptRNA^{Thr}$ or $ptRNA^{Ser}$ of 5 μM in 30 μl, we need:

24 mer = 0.67 μl

59 mer (20% excess) = 3.53 μl

5x modified PA = 6 μl

dd H_2O = 19.8 μl

Total = 30 μl

Buffer used in the standard 1x PA + all additives.

* The final enzyme concentration in each well was ~12 nM. For either substrate that was no discernable change in polarization as a function of time.

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pRNA-Leu Time (min)	[S]=100 nM, [E]=12 nM	[S]=100 nM, [E]=0 nM	[S]=50 nM, [E]=12 nM	[S]=50 nM, [E]=0 nM	[S]=25 nM, [E]=12 nM	[S]=25 nM, [E]=0 nM	[S]=12.5 nM, [E]=12 nM	[S]=12.5 nM, [E]=0 nM
0	270	272.5	279	272.875	285.05	285.425	281.725	290.975
2.5	262.25	268.975	270.8	269.525	274.175	281.025	278.45	285.25
5	259.45	265.25	267.275	267.925	273.775	276.725	269.175	288.525
10	252.55	260.7	263.925	263.9	268.6	276.375	266.3	286.025
15	246	259.35	258.625	262.625	265.475	272.875	263.65	287.525
25	245.2	257.525	255.825	263.725	263.25	274.325	263.025	287.4
35	242	255.875	253.65	265.15	262.95	275.975	263.025	289.875
45	242.275	257.625	255.25	265.775	264.8	280.7	266.875	293.425
60	246.525	261.5	254.475	270.625	261.975	283.2	268.825	292.475

pRNA-Thr Time (min)	[S]=100 nM, [E]=12 nM	[S]=100 nM, [E]=0 nM	[S]=50 nM, [E]=12 nM	[S]=50 nM, [E]=0 nM	[S]=25 nM, [E]=12 nM	[S]=25 nM, [E]=0 nM	[S]=12.5 nM, [E]=12 nM	[S]=12.5 nM, [E]=0 nM
0	272.275	284.45	274.25	289.85	283.875	293.75	283.8	301.2
2.5	262.625	281.25	271.325	289.2	277.6	292.075	280.775	295.825
5	262.375	281.7	267.125	287.175	277.125	292.025	281.325	295.825
10	264.85	278.075	267.95	282.825	276.8	292.9	280.05	293.65
15	258.925	275.1	265.725	281	275.55	287.675	281	291.075
25	256.7	271.2	264.05	275.625	272.25	283.175	278.175	288.05
35	254.025	265.675	261.3	273.125	270.625	279.15	274.325	286.65
45	253.85	261.525	257.175	271.95	269.975	280.6	276.6	284.275
60	247.675	259.575	255.675	271.025	269.1	278.775	274.025	286.125

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RNase P renaturation:

RNA M1 = 2 µl

10X PA = 4 µl

dd H_2O = 28 µl

Heat at 65°C, 55°C and 37°C for 5' each.

Add, Protein = 2 µl

DTT = 4 µl

Renature at 37°C for 5 minutes.

Final concentration of RNase P ≈ 1 µM.

ptRNA^{Thr} renaturation:

24 mer = 1.34 µl

59 mer (20% excess) = 7.66 µl

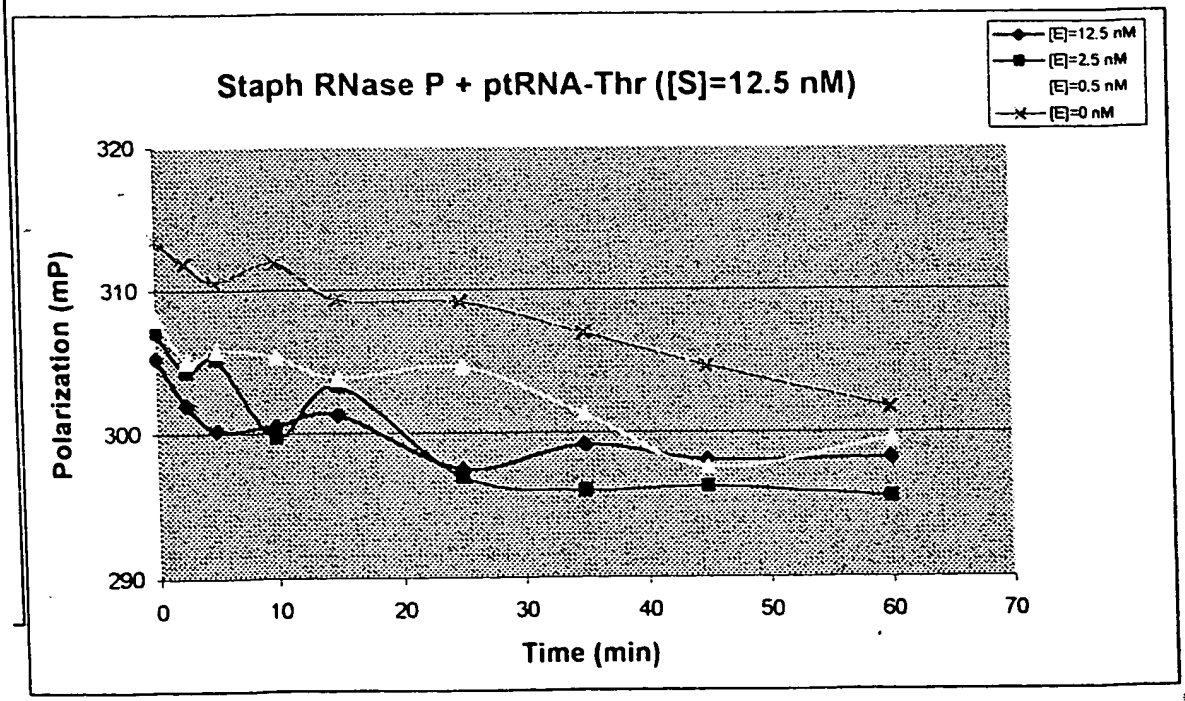
10X PA = 6 µl

dd H_2O = 45.6 µl

Renature by heating at 65°C, 55°C and 37°C for 5 minutes each

Three enzyme concentrations 0.5 nM, 2.5 nM and 12.5 nM, along with three ptRNA^{Thr} concentrations 12.5 nM, 25 nM and 50 nM were [S]=25 nM = 1X PA buffer with additives was used

Time (min)	[E]=12.5 nM	[E]=2.5 nM	[E]=0.5 nM	[E]=0 nM
0	305.3	307.15	308.05	313.575
2.5	302.05	304.425	305.1	311.95
5	300.3	305.25	305.825	310.6
10	300.6	299.85	305.375	312
15	301.35	303.375	303.875	309.375
25	297.625	297.025	304.625	309.2
35	299.225	296.075	301.3	307.05
45	298.225	296.35	297.625	304.775
60	298.325	295.6	299.575	301.85



to Page 112

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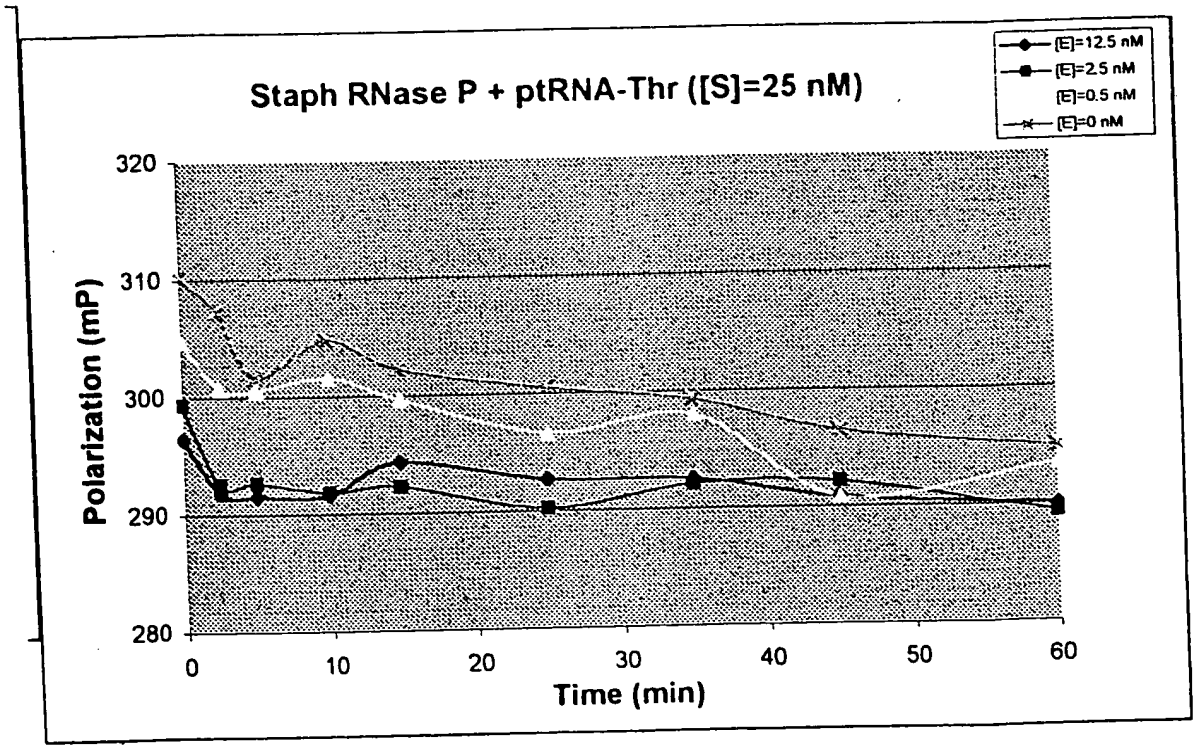
DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

Work continued from Page 111

[S]=25 nM

Time (min)	[E]=12.5 nM	[E]=2.5 nM	[E]=0.5 nM	[E]=0 nM
0	296.525	299.4	304.675	310.275
2.5	291.875	292.65	300.7	307.4
5	291.575	292.725	300.55	301.775
10	291.7	291.925	301.6	304.925
15	294.4	292.325	299.65	302.35
25	292.8	290.325	296.625	300.6
35	292.65	292.175	298	299.425
45	290.925	292.225	290.85	296.625
60	290.1	289.475	293.5	295.025



Work continued to Page 113

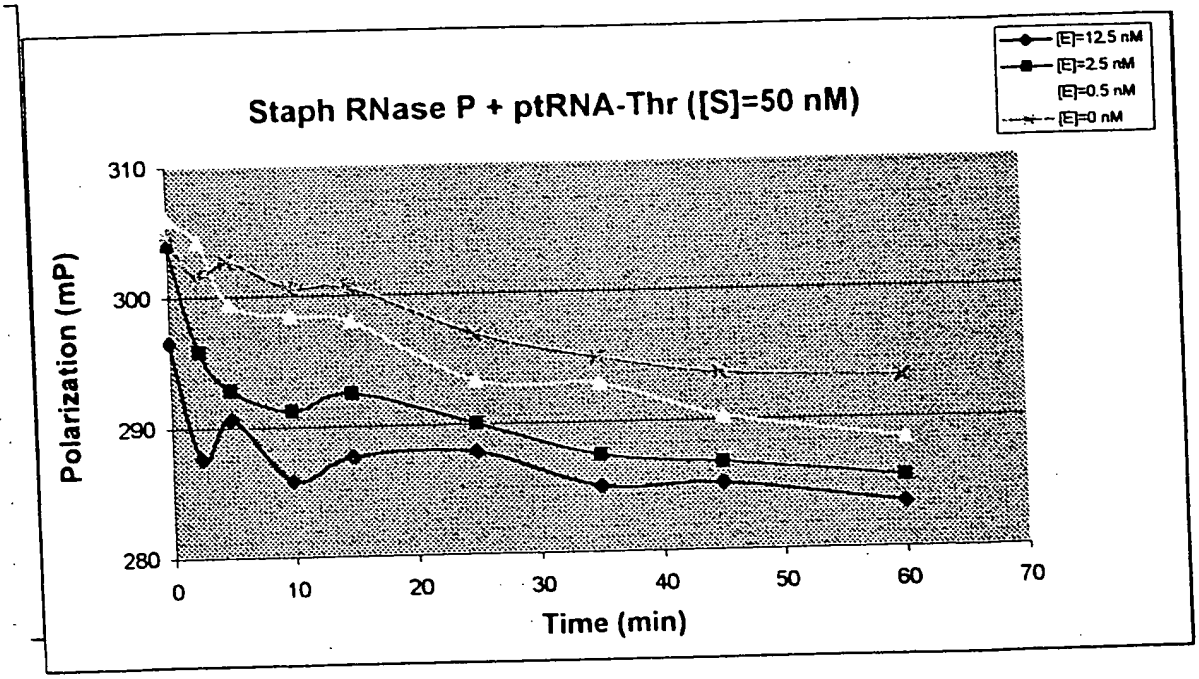
SIGNATURE K Anish Xavier DATE

DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

Work continued from Page 112

[S]=50 nM

Time (min)	[E]=12.5 nM	[E]=2.5 nM	[E]=0.5 nM	[E]=0 nM
0	296.6	304.1	306.05	304.775
2.5	287.65	295.925	304.25	301.675
5	290.675	292.9	299.425	302.825
10	285.875	291.25	298.55	300.75
15	287.725	292.625	298.025	300.725
25	287.925	290.1	293.275	297.025
35	285.075	287.525	292.9	295.025
45	285.2	286.8	290.3	293.675
60	283.525	285.525	288.4	293.225



Based on the results, the staph RNase P enzyme does cleave the annealed ptRNA^{Thr}; however the decrease in measured polarization is insufficient to configure our assay with. Therefore, we are going to try higher concentrations of both Staph RNase P and the Staph substrate ptRNA

Work continued to Page

SIGNATURE K Anish Xavier

DATE

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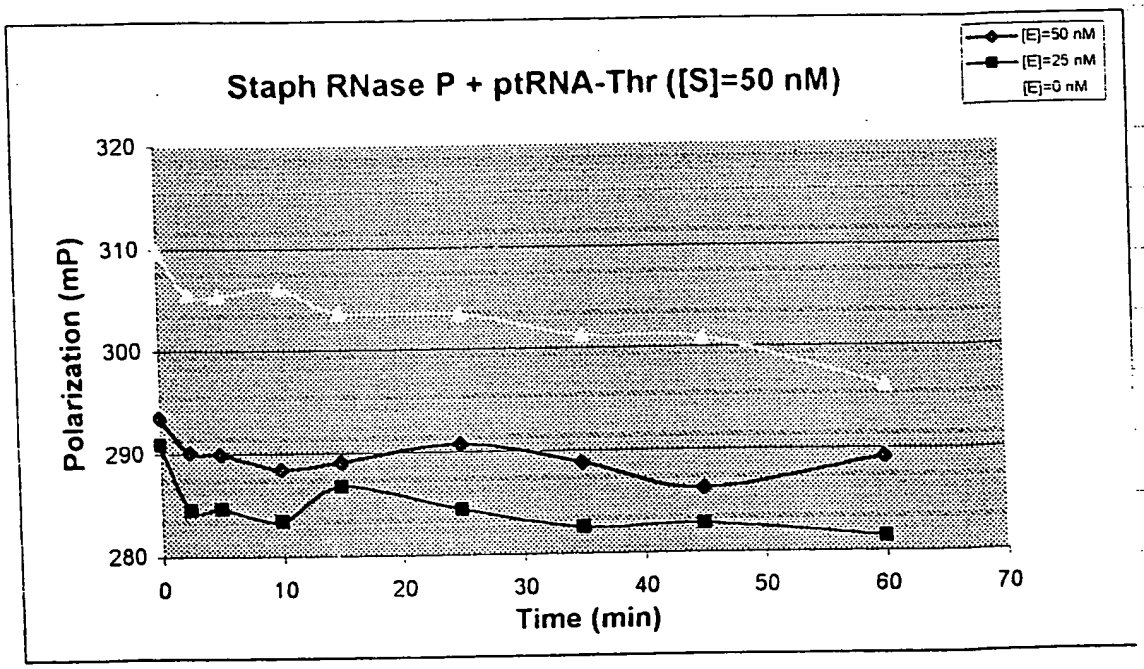
The previous experiment was repeated at Staph RNase P concentrations of 25 nM and 50 nM, with both substrates $ptRNA^{Thr}$ and $ptRNA^{Leu}$. The substrate concentrations tried were 50 nM and 100 nM.

ptRNA-Thr
[S]=50 nM

Time (min)	[E]=50 nM	[E]=25 nM	[E]=0 nM
0	293.63333	290.96667	309.7667
2.5	290.16667	284.6	305.5333
5	289.96667	284.7	305.3
10	288.53333	283.53333	306
15	289.16667	286.83333	303.6333
25	290.83333	284.53333	303.4667
35	288.93333	282.63333	301.2333
45	286.33333	282.9	300.9667
60	289.23333	281.53333	296.1667



At E[ptRNA^{Thr}]=50 nM and RNase P concentrations of 25 nM and 50 nM, there was no significant change in polarization, which could be attributed to enzyme cleavage.

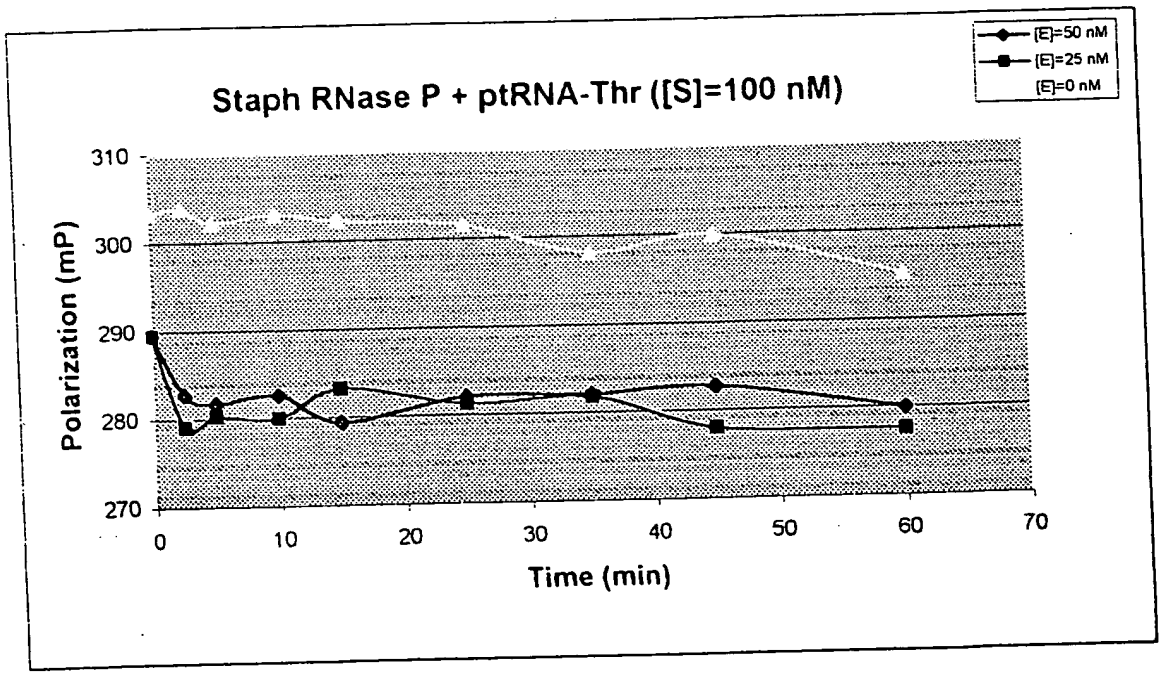
SIGNATURE K Anish Xavier DATE

DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

Work continued from Page 114

ptRNA-Thr
[S]=100 nM

Time (min)	[E]=50 nM	[E]=25 nM	[E]=0 nM
0	289.633333	289.56667	303.3
2.5	282.866667	279.1	303.9
5	281.833333	280.36667	302.1667
10	282.666667	280.1	303.0667
15	279.5	283.36667	302.2333
25	282.166667	281.4	301.5667
35	282.2	281.86667	298.1333
45	282.833333	278.1	299.9333
60	280.066667	277.63333	295.2



Again, at [$ptRNA^{Thr}$] = 100 nM and RNase P concentrations of 25 nM and 50 nM, there was no significant change in measured polarization, that could be attributed to enzyme cleavage.

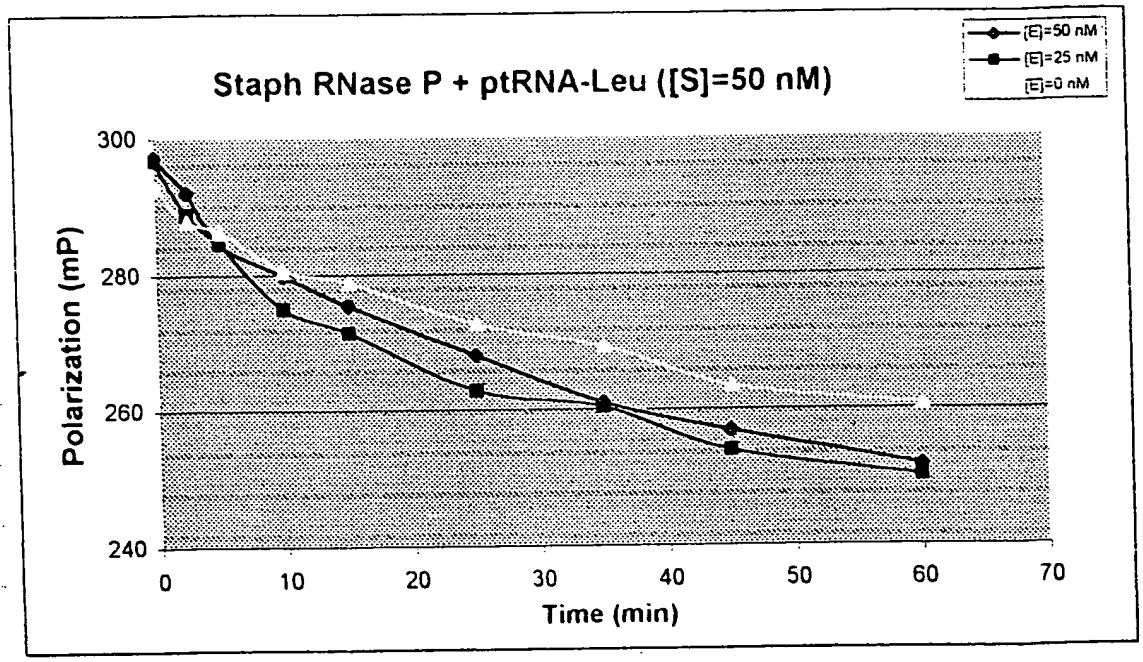
SIGNATURE K Anish Xavier DATE

DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

Work continued from Page 115

ptRNA-Leu
[S]=100 nM

Time (min)	[E]=50 nM	[E]=25 nM	[E]=0 nM
0	297.4	296.86667	292.7667
2.5	292.16667	288.96667	287.9
5	284.73333	284.7	286.4
10	279.93333	275.06667	280.5333
15	275.5	271.56667	278.7
25	268.16667	262.93333	272.5667
35	261.13333	260.46667	269.2667
45	256.96667	254.06667	263.4333
60	251.86667	250.26667	260.6333



At [ptRNA^{Leu}] = 50nM and RNaseP concentrations of 25nM and 50 nM, there was no significant change in measured polarization, which could be attributed to enzyme cleavage.

Work continued to Page 117

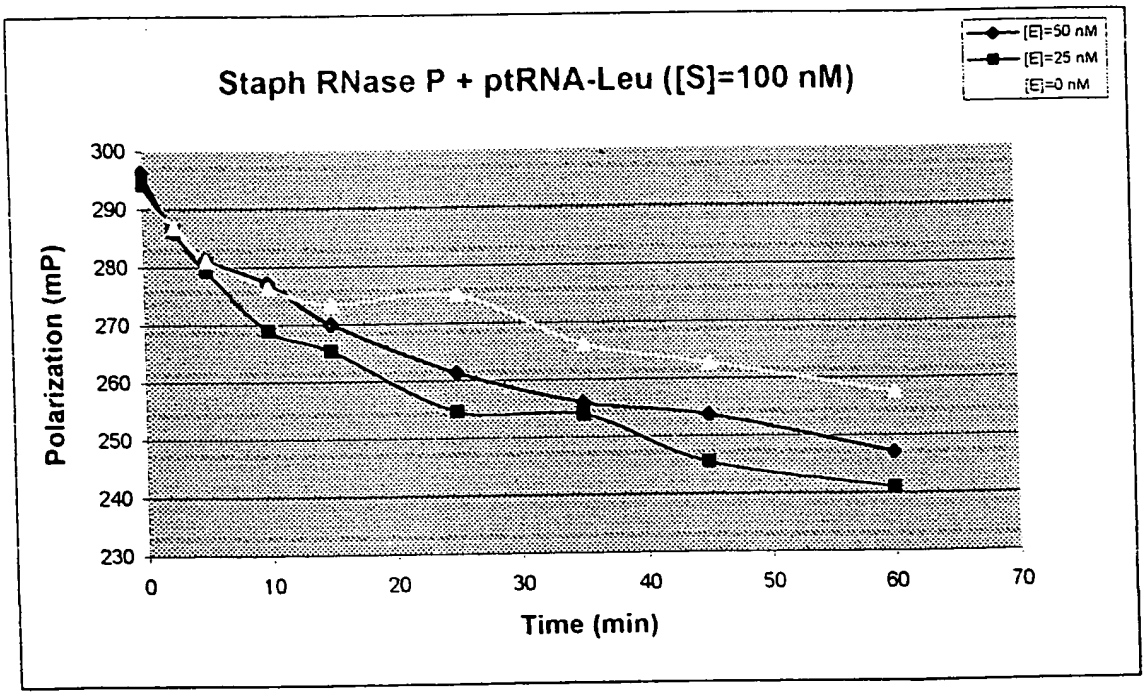
SIGNATURE K Anish Xavier DATE

DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

Work continued from Page 116

ptRNA-Leu
[S]=100 nM

Time (min)	[E]=50 nM	[E]=25 nM	[E]=0 nM
0	296.53333	294.33333	291.8
2.5	286.06667	286.36667	286.9333
5	281.4	279.36667	281.1667
10	277.26667	269.1	276.3667
15	270.2	265.53333	273.3667
25	261.6	254.8	274.8333
35	256.13333	254.16667	266.1333
45	253.9	245.73333	262.7
60	247.3	241.16667	257.5333



At $[E_{ptRNA^{Leu}}]$ = 50 nM and RNase P concentration of 25 nM and 50 nM, there was no significant change in measured polarization, which could be attributed to enzyme cleavage.

Given the poor performance of Staph substrates ptRNA^{Thr} and ptRNA^{Leu}, it was decided to test ptRNA^{Gln} with Staph RNase P.

Renaturation:

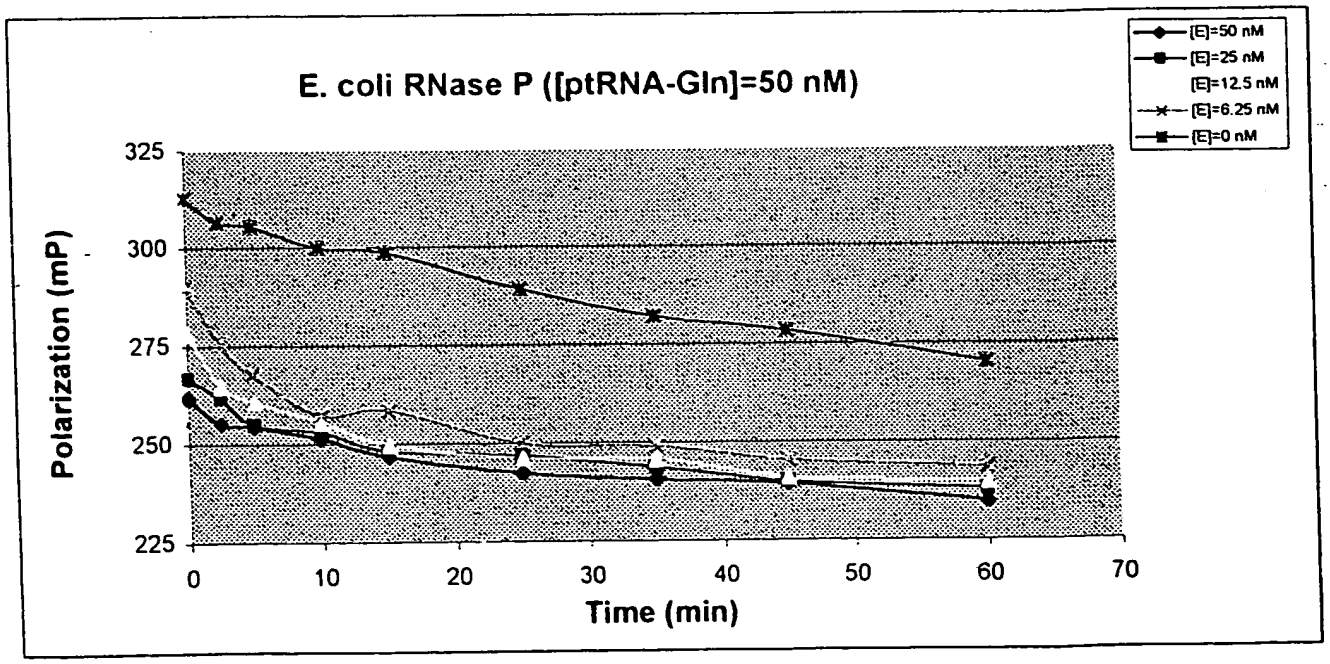
1) E. coli RNase P (final conc. = 500 nM)

M1 RNA = 1.5 µl
10 x PA = 4.5 µl
dd H_2O = 30 µl
Protein CS = 4.5 µl
DTT = 4.5 µl

2) Staph RNase P (final conc. = 1 µM)

M1 RNA = ~~2.0~~ µl
10 x PA = ~~4.0~~ µl
dd H_2O = 25 µl
Protein CS = 2 µl
DTT = 4 µl

Time (min)	[E]=50 nM	[E]=25 nM	[E]=12.5 nM	[E]=6.25 nM	[E]=0 nM
0	261.925	266.825	277.9	290.175	313.025
2.5	255.425	261.85	264.65	275.675	306.725
5	254.575	255.1	260.5	267.675	305.65
10	251.375	253.275	255.6	257.55	300.15
15	246.75	248.225	249.7	258.65	298.925
25	242.525	246.975	246.225	250.15	289.45
35	240.75	243.825	245.625	249.225	282.3
45	239.475	240.275	241.025	245.725	278.575
60	234.975	238.075	239.7	243.7	270.45



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Work continued from Page 115

3) $pt RNA^{Gln}$ (final conc. = 5 μM)

26mer (150 μM) = 1.7 μl

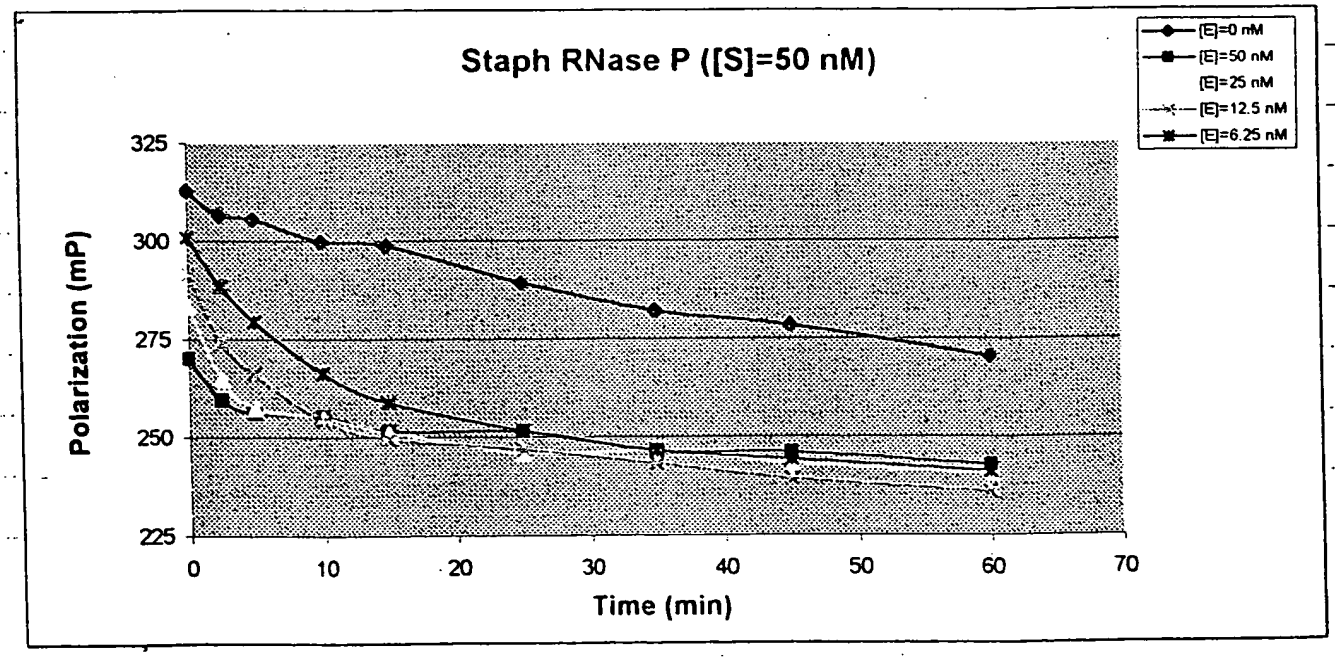
59mer (250 μM) = 1.3 μl (20% excess)

10 X PA = 5 μl

dd H_2O = 42 μl

Total = 50 μl

Time (min)	[E]=0 nM	[E]=50 nM	[E]=25 nM	[E]=12.5 nM	[E]=6.25 nM
0	313.025	270.2	283.025	291.75	301.1
2.5	306.725	259.725	264.625	274.225	288.7
5	305.65	256.3	257.45	266.075	279.675
10	300.15	255.425	255.175	254.45	266.4
15	298.925	251.675	251.25	249.7	259.05
25	289.45	251.625	245.825	247.15	251.825
35	282.3	246.525	246.25	243.5	246.725
45	278.575	246.325	243.225	239.6	244.55
60	270.45	243.025	240.425	236.125	241.375



⇒ $pt RNA^{Gln}$ is an effective substrate when used with Staph RNase P at a substrate concentration of 50 nM and enzyme concentrations of 0.25 nM, 12.5 nM, 25 nM & 50 nM.

120 TITLE Testing 5'-TAMRA Labeled $ptRNA^{Gln}$ PROJECT NO.

Work continued from Page ____ BOOK NO.

$ptRNA^{Gln}$ was in vitro transcribed (using protocol from Altman laboratory) using 5:1 excess of thiolated GMP over GTP. This resulted in incorporation of GMP in the synthesized $ptRNA^{Gln}$. The transcription mixture was treated with DNase and then subjected to phenol-chloroform extraction.

The RNA was ~~reacted with~~ precipitated with sodium acetate and isopropanol and then resuspended in 20 mM sodium phosphate (pH = 7.5). The RNA was reacted with TAMRA iodoacetamide (in DMF) at at least 5 molar excess for two hours at room temperature. The reaction was quenched by adding excess of DTT. The reaction mixture was dialyzed (in a Pierce 500 µl slide-a-lyzer cartridge) with multiple changes of buffer.

~50 nM $ptRNA^{Gln}$ ~~it was~~ with various concentrations of Staph. RNase P (2.5 nM, 7.5 nM and 30 nM) was tested. It was observed that the measured polarization was too low (~40 mP), though there was sufficient counts ~35 million cps.

One hypothesis was that the TAMRA had no linker and a linker may be required for significant measure polarization. For this reason, BODIPY iodoacetamide (ultimate attachment) (will have a stiff linker), was ordered from Molecular Probes.

To make sure intercalation, TAMRA 5' labeled $ptRNA^{Gln}$ will be gel purified.

Work continued to Page ____

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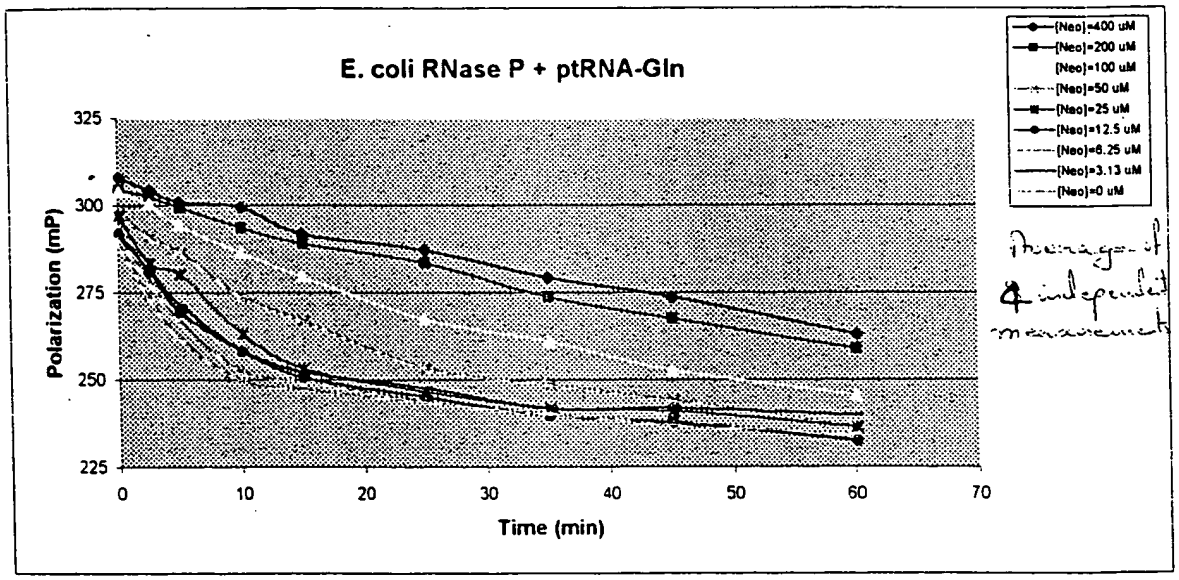
To check whether the observed difference of ~50 mP was sufficient to configure an assay for RNase P cleavage, we checked the affe of Neomycin B on E. coli and Staph RNase P (in the concentration 3.13 μM to 400 μM).

5

Time (min)	[Neo]=400 uM	[Neo]=200 uM	[Neo]=100 uM	[Neo]=50 uM	[Neo]=25 uM	[Neo]=12.5 uM	[Neo]=6.25 uM	[Neo]=3.13 uM	[Neo]=0 uM
0	308.075	304.425	303.95	297.725	296.875	291.975	290.8	291.7	288.975
2.5	304.175	302.5	300.425	291.075	283.325	281.1	277.95	281.275	273.325
5	301	299.35	294.325	286.5	280.2	269.325	266.1	271.375	261.6
10	299.5	293.55	286.625	273.85	263.225	258.1	252.175	258.275	249.675
15	291.775	288.9	279.65	266.925	253.05	250.575	249.45	252.225	247.275
25	287.2	283.525	267.425	253.35	246.425	245.05	243.775	247.55	243.65
35	279.2	273.7	261.075	248.5	241.85	239.625	239.7	241.775	240.225
45	273.8	267.5	252.8	244.075	241.075	237.675	238.075	241.85	236.925
60	262.975	258.775	245.525	236.45	236.325	232.375	233.125	239.925	234.125



Average of 4 independent measurement

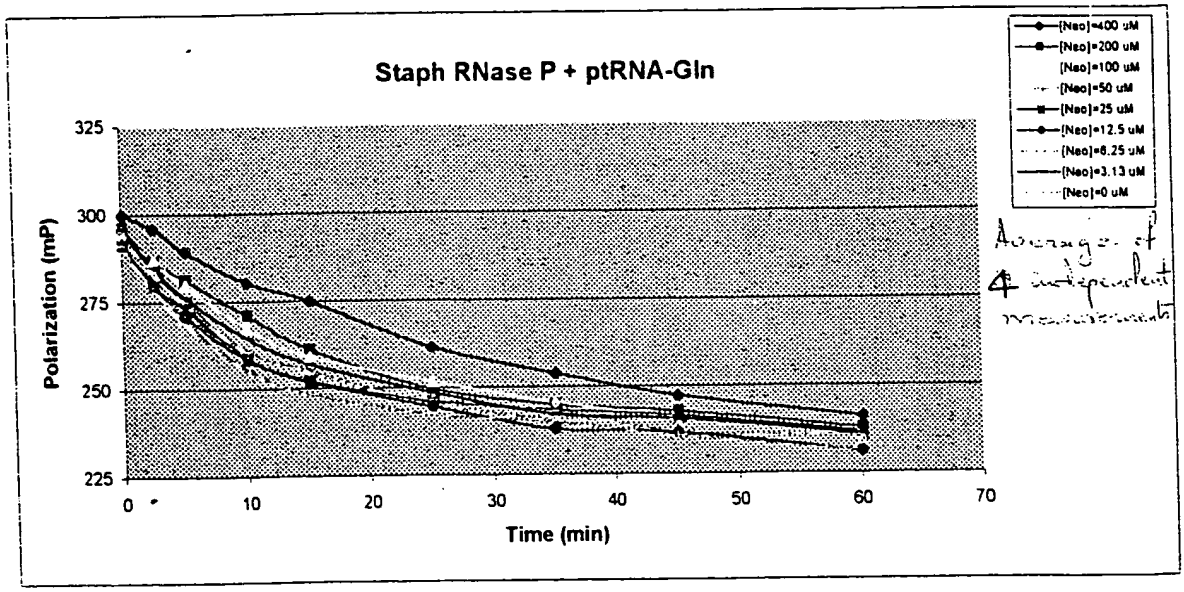
Based on the time course, the EC_{50} is between 50–100 μM Neom B for E. coli RNase P. This is in agreement with published l

25 above data.

SIGNATURE K Arab Xavier DATE

DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

Time (min)	[Neo]=400 uM	[Neo]=200 uM	[Neo]=100 uM	[Neo]=50 uM	[Neo]=25 uM	[Neo]=12.5 uM	[Neo]=6.25 uM	[Neo]=3.13 uM	[Neo]=0 uM
0	299.975	296.3	295.275	295.6	291.6	291.15	291.075	295.95	291.425
2.5	295.975	286.65	286.875	283.475	279.65	279.7	279.075	284.9	279
5	289.3	281.175	279.725	274.825	273.1	270.825	271.2	276.125	269
10	280.375	270.95	267.55	260.725	258.8	258.2	258.925	264.4	255.275
15	275.075	261.275	259.075	254.95	252	251.925	252.575	257	248.325
25	261.65	249.7	251.825	245.55	246.05	244.4	246.55	248.6	242.275
35	253.9	244.875	245.5	240.7	243.3	237.875	243.025	242.15	239.875
45	247.275	242.8	241.125	240.875	241.625	236.725	236.875	240.575	235.925
60	241.2	237.9	236.825	235.625	236.75	231.05	235.575	236.025	231.275



Average of 4 independent measurements

For Staph RNaseP only the highest Neomycin B concentration of 400 μM has any inhibitory effect.

Based on the Neomycin B data, as long as the variability across a plate is minimal, it should be possible to configure a first generation screen.

BODIPY labeled ptRNA^{Gln} (using 5' thiolated GMP and iodoacetamide-BODIPY) was gel purified and the appropriate gel band subjected to "crush and soak". The recovered material was serially diluted and its polarization determined. The measured polarization was about 100 - 110 mP, and the intensity at the higher concentrations was adequate and gave a few million count/second.

Some of this material was also subjected to Staph RNase P cleavage (at an enzyme concentration of ~25 nM). No change in measured polarization as a function of time was observed.

Note, a complicating factor is the presence of significant amount of urea in the BODIPY labeled ptRNA^{Gln} from the "crush and soak" procedure.

SIGNATURE K Anish Xavier DATE

DISCLOSED TO AND UNDERSTOOD BY | DATE | WITNESS | DATE

124 TITLE Testing 5'-BODIPY labeled ptRNA^{Gln} PROJECT NO.

BOOK NO.

Work continued from Page ____

The 5'-BODIPY labeled ptRNA^{Gln} was diluted with many changes of buffer. The sample was serially diluted and the polarization measured using filters used with TAMRA. The measured polarization was 30-40 mP, which is too low.

Given that intercalation could be problem and therefore gel purification may be needed this which give yields that are too low to be useful for HTS. Hence, this approach ~~was~~ is going to be discontinued.

The 5'-TAMRA labeled ptRNA^{Gln} was gel-purified and recovered by "crush and soak" The ~~s~~material was serially diluted and the polarization measured. The measured polarization was between 120 and 150 mP, ~~which is~~ with sufficient intensity. This indicates that the previously observed low polarization measurement was at least partially due to intercalation of the TAMRA.

SIGNATURE K Anish Xavier DATE

DISCLOSED TO AND UNDERSTOOD BY | DATE | WITNESS | DATE

The assay was configured in a 384-well format with a volume of 30 µl/well (10 ul of 30 µM compound + 10 µl of 30 nM Staph RNase P + 10 µl of 150 nM bipartite substrate). It was observed that there was substantial loss of signal during the 10-12 minutes required for enzyme cleavage.

Repeating the same experiment by hand reproduced the results, suggesting that the observation did not have anything to do with the Multimek formatting of the assay.

When the same setup (in terms of component concentration was repeated with 96-well plates using the Multimek or by hand it was observed that there was sufficient signal to develop an assay for the first 15 or so minutes. The only difference in the 96-well plates is the larger volume of 100 µl.

Note: signal intensity was not an issue in either 384-well or 96-well format.

To overcome the problem(s) of signal loss, 384-well and 96-well plates were coated with the buffer (with all additives) and then used after about 2 hours (the buffer was removed by vigorously flicking the plates). The coating did not have any affect on the loss of signal/measured polarization.

SIGNATURE K Anish Xavier DATE

DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

126 TITLE Testing 96-well format for Screening.

PROJECT NO.

BOOK NO.

Work continued from Page ____

The assay was configured in a 96-well format (total volume of 30 µl/well). Columns 1-3 had no enzyme (only substrate), 4-6 had both enzyme and substrate and column 7 had only buffer.

Plate-1

A	280.9	283.1	284.1	255.1	253.5	251	691
B	278.7	284	286.2	255.8	255.9	251.5	638.8
C	282.7	288.4	282.3	257.2	247.2	253.7	614
D	279.2	282.8	288.2	253.8	253.1	251.7	623.9
E	281.7	281.2	285.1	252.3	258	248.4	609.7
F	281.3	283.2	280.8	252.6	253.4	246.9	628.4
G	280.1	279.3	281.3	249.7	252.9	248.4	682.7
H	277.2	277	275	249.7	248.9	251.8	580.1
			281.825			252.1875	
			3.291788			3.027456	

Plate-2

A	282.8	277.8	282.9	242.6	246.8	244.6	632.8
B	275.2	278.5	280	248.8	247.1	243.1	649.4
C	278.2	272.8	280.3	243.7	245.5	246.3	591.4
D	276.6	274.5	282.8	246.3	242	244.4	590.3
E	275.9	278.2	279.9	244.6	243	244.6	649.9
F	276.6	273.8	274.5	244.4	248.5	242.8	580.1
G	274.7	280.3	271.8	246	244.5	246	604.7
H	273.1	275.7	271.9	244.2	242.7	245.2	539
			277.0333			244.9042	
			3.40979			1.818618	

Plate-3

A	260.2	268.6	268.8	236.2	231.6	233.6	668.5
B	259.9	267	267	234.8	232.9	231.5	647.2
C	263.8	262.3	261.7	231.8	229.7	226.3	709.8
D	263.9	267.5	258.8	229.5	231.1	231.4	609.7
E	263.7	262.6	262.4	230.5	232	226.8	635.5
F	265.1	266.9	259.9	234.4	232	265	648.9
G	263.1	260.3	262.2	232	233.4	230.4	668.5
H	260.1	264.6	262.4	232.1	234.5	234.6	535.5
			263.45			233.2542	
			2.953406			7.157664	

Thus, using the Multimek there was a consistent difference of ~30 mP in the presence of enzyme. We will try to configure our assay using this difference.

Work continued to Page ____

SIGNATURE K Anish Xavier DATE

DISCLOSED TO AND UNDERSTOOD BY DATE WITNESS DATE

Buffer: 50 mM Tris-HCl (pH = 7.5), 100 mM NH_4Cl, ~~ie~~ nucleotides with 2% glycerol, 25 µg/mL carbonic anhydrase, 5 µg/mL tRNA.

Each well will have 80 µl (20 µl of 40 µM compound + 40 µl of enzyme + 20 µl of 200 nM substrate)

⇒ Experiments with renatured substrate showed that once the substrate was diluted from 5 µM to 200 nM, there was no change in the effect. Therefore it was decided to freshly dilute substrate for each screening plate.

On ~~two~~ plates were screened to work out the kinks in the screening procedure. It was observed that a lot of substrate was wasted when using "deep-well" reservoirs, hence they were replaced with "flat-bottom" 96-well plates.

On screening results were encouraging but there were many anamolous values due to air bubbles (due to lack of sufficient enzyme and substrate)

We believe all kinks have been worked out to be able to screen in 96-well format.

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